CELLULAR AND MOLECULAR MECHANISMS THAT REGULATE OLFACTORY RHYTHMS IN *DROSOPHILA MELANOGASTER*

A Dissertation

by

PARTHASARATHY KRISHNAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Biology

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Approved by:

Chair of Committee,	Paul Hardin
Committee Members,	Vincent Cassone
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ABSTRACT

Cellular and Molecular Mechanisms That Regulate Olfactory Rhythms in *Drosophila melanogaster.* (May 2008)

Parthasarathy Krishnan, B.S., University of Madras; M.S., University of Madras

Chair of Advisory Committee: Dr. Paul Hardin

This dissertation is focused on how circadian control of olfactory responses are regulated at the cellular and molecular level in *Drosophila*. Electrophysiological approaches consisting of Electroantennogram (EAG), single unit recordings, among other techniques, were used to investigate the extent of autonomy of peripheral oscillators from central pacemaker cells, the molecular targets of the circadian oscillator in antennal neurons, and the nature of circadian influence on single unit responses recorded from basiconic sensillae.

To address the question of cellular mechanisms mediating olfaction rhythms, UAS-Gal4 strategies were used for tissue specific expression of dominant negative forms of CLK (CLOCK) and CYC (CYCLE). Specifically, OR (Odorant Receptor)-GAL4 constructs were used to achieve cell specific expression in the antenna. By recording EAG responses from specific regions of the antenna, it was found that antennal sensory neurons possess independent oscillators that are both necessary and sufficient to drive rhythms in olfactory responses.

To understand the molecular mechanisms controlling olfaction rhythms, the effect of GRK-2 and an arrestin (KURTZ) of the olfactory signal transduction pathway were studied by use of respective mutants and the effect of cell specific rescue of these proteins by UAS-Gal4 approaches was also documented. Interestingly, these molecules have phenotypes that argue for a different role of for these proteins in *Drosophila* olfaction as opposed to their respective functions in vertebrate systems.

Finally, single unit recordings were measured from different basiconic sensilla and the influence of the circadian oscillator was studied on select parameters of the single unit data obtained. It was found that spike amplitude of the spontaneous response was the only parameter under circadian control and that these rhythms are dependant on input from the odorant receptor activated pathway.

DEDICATION

I dedicate this dissertation to Appa and Amma, who have given me literally and metaphorically ‘the eyes to see’.

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I would like to thank my committee chair, Dr. Paul Hardin, who has been of much help to me during my time as a graduate student. From Paul, I have learned to appreciate *Drosophila* as a model organism and increased my critical writing skills. I thank Dr. Stuart Dryer, who molded much of my thinking as an electrophysiologist and for helping me set up electronic equipment for performing EAG recordings. I thank the rest of my committee members Dr. Mark Zoran, Dr. Vincent Cassone, and Dr. Gladys Ko for helpful advice along the way of my graduate career. While at the University of Houston, I received much help from Dr. Costa Colbert and Dr. Gregg Roman.

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Living thousands of miles away from family and friends is no joke and the following people made me feel very much at home and get accustomed to life in the

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Finally, thanks to my parents and my brother for their love and support throughout my time in the United States. Also, I am indebted to my wife Sushma, who has made everything look worthwhile to me.

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CHAPTER I

GENERAL INTRODUCTION

OVERVIEW

Change is universal in nature. Rhythmic events, both biological and non-biological have captured man's curiosity and fascination. Diverse life forms have evolved on Earth, with an internal biological clock that is tightly linked with the daily rhythmic events of sunrise and sunset. These biological rhythms are referred to as circadian (circa-about; diem-day). Circadian clocks influence numerous behavioral and physiological processes that aid in the survival of organisms that range from bacteria to vertebrates. The fundamental molecular processes driving these rhythms remain remarkably conserved across different phyla (Yu and Hardin, 2006). The components of these time keeping systems can be roughly categorized into 1. Input/entrainment pathway, 2. core molecular oscillator and 3. An output pathway, which ultimately drives physiological and behavioral rhythms. Though components of the core oscillator are well understood in *Drosophila*, the organization of circadian clocks (hierarchy) and how the clock communicates time to drive rhythmic behavioral and physiological processes are topics that still need much understanding.

This dissertation follows the style of Cell.

FORMAL PROPERTIES OF CIRCADIAN RHYTHMS

A host of criteria serve as a yardstick in determining whether an oscillation in a biological system is indeed circadian in nature. By definition, a process under clock control must display a period of oscillation, which is approximately twenty-four hours and persist in the absence of environmental time cues. The term *free-run* is used to describe the persistent nature of the rhythm under absence of external environmental cues or *zeitgebers (*time givers in German) (Pittendrigh, 1960). By conferring anticipation of local environmental time, oscillators provide a temporal coordination of the organism's internal process with that of the external environmental cycles (Bell-Pederson et al., 2005). In natural conditions, the light-dark cycles entrain oscillator driven processes and serve as *zeitgebers* capable of regulating both the period and phase relationships of circadian rhythms. In other words, photic entrainment requires the constant adjustment of the endogenous period of the organism so that it almost equals the period of the entraining zeitgeber. Thus, through both period and phase control of rhythmic processes, circadian entrainment ensures that critical biological processes are coordinated with local environmental times. Another formal property of biological clocks is that the period of the circadian rhythm is temperature compensated (Kurosawa and Iwasa, 2005). The maintenance of the intrinsic circadian period across a range of temperatures is thought to maintain a temporal synchronization among different clock controlled processes. This phenomenon was first described in *Drosophila* (Pittendrigh, 1954) and has been found true in numerous other species. These formal properties are

necessary for faithful translation of biological oscillations into behaviorally meaningful patterns that serve to adapt an organism to changing environmental conditions.

THE *DROSOPHILA* CIRCADIAN SYSTEM

The circadian system in *Drosophila* is composed of interlocked feedback loops in gene expression, in which certain genes are negatively regulated by their own gene products (Hardin, 2005). In *Drosophila*, the oscillator is comprised of two interlocked negative feedback loops (Figure 1): CLK and CYC, which bind E box elements to transcriptionally activate *vrille (vri), PAR domain protein Ic* (*pdp1c*), *period (per)*, and *timeless(tim)*. VRI and PDP1c feedback to repress and activate Clk transcription, respectively, while PER and TIM feedback as a complex with DOUBLETIME (DBT) kinase to repress CLK-CYC dependant transcription (Cyran et al., 2003). These interlocked feedback loops maintain rhythmic cycles in gene expression that control circadian outputs. Light dependant phase shifting or entrainment is achieved by activation of a photoreceptor, which then alters the level/activity of an oscillator component. In *Drosophila,* several photoreceptors are activated by light and this is the trigger for the degradation of Tim (Timeless) (Hardin, 2005).

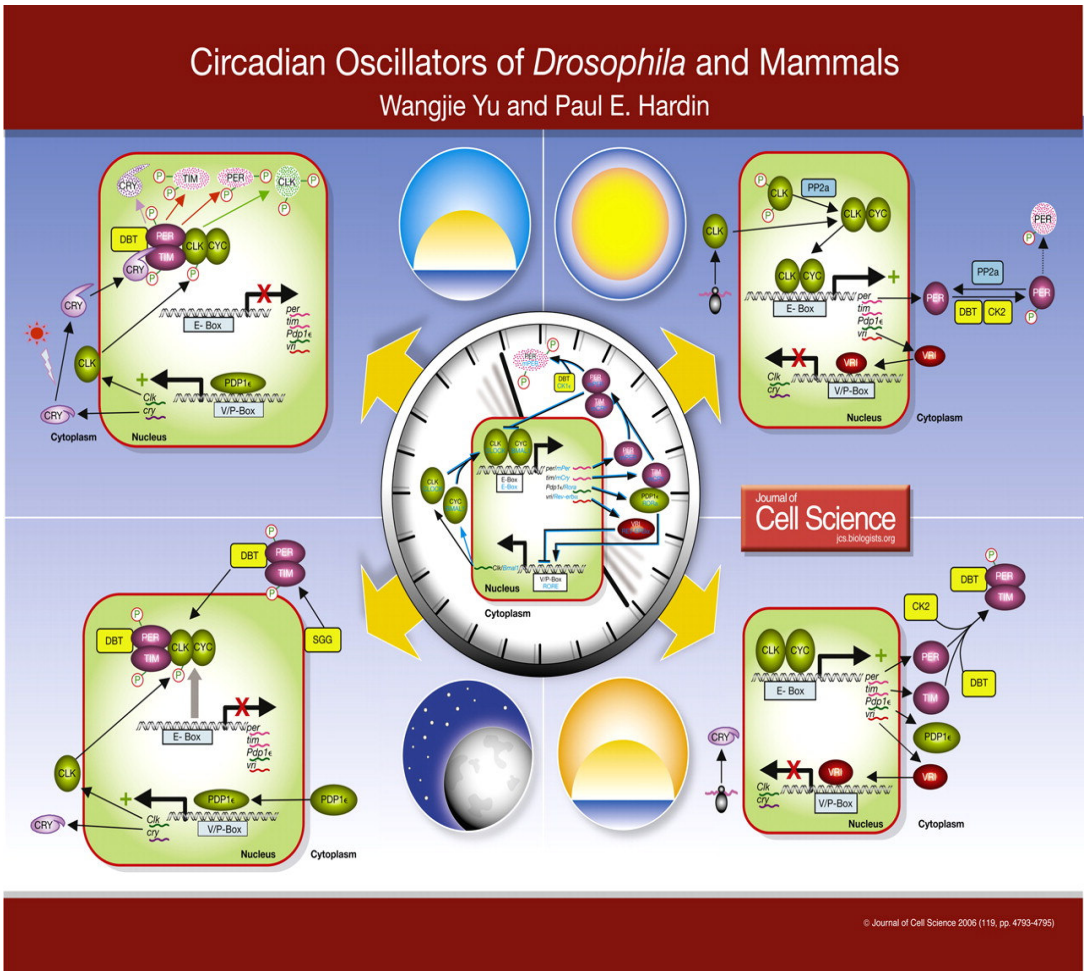

Figure 1: Molecular components of circadian oscillators. (Figure reproduced from Yu and Hardin, 2006.)

Although much is known about circadian oscillator function in *Drosophila,* relatively little is known about the output pathways that mediate rhythmic physiological and behavioral processes. In fruit flies, circadian oscillators are present in diverse tissues throughout the adult body. Using *per* driven luciferase reporter genes, it has been shown that these oscillators operate independently and they are directly light entrainable (Plautz et al., 1997). Also, oscillators in cultured *Drosophila* tissues, can be directly entrained by light indicating that they operate in a tissue autonomous way (Hardin, 2005). This model of oscillator function is distinct from the hierarchical nature of mammalian systems.

Locomotor activity rhythms have been the most widely characterized rhythmic process in *Drosophila*. The small ventral lateral neurons (sLNv's) in the brain rhythmically produce PDF (Pigment Dispersing Factor), which mediates free running locomotor activity rhythms. Also when the large and small LNv's are electrically silenced by expressing a mutant K^+ channel causes the elimination of Per and Tim molecular oscillations (Nitabach et al., 2002). This suggests that electrical activity is necessary for oscillator function in these cells. Rhythm in eclosion is another characterized behavioral output rhythm. This rhythm is 'semi independent', in that input from both the peripheral oscillator (prothoracic gland) and the LNv's are required to sustain the rhythm (Myers et al., 2003). Thus communication between these two oscillator cells is important for sustaining rhythms in olfaction. One other physiological rhythm that will be discussed in detail in this dissertation is the rhythm in EAG (Electroantennogram) responses (summed receptor potentials) in response to food

odorants (Krishnan et al., 1999). These responses are at their peak in the middle of the subjective night and minimum during the day and at the end of the night. This rhythm persists under free running conditions and is absent in *period* and *timeless* mutants.

ORGANIZATION OF THE *DROSOPHILA* OLFACTORY SYSTEM

Overview

The sense of smell is probably the oldest sensory system in the animal kingdom (Hildebrand and Shepherd, 1997). Although perhaps not entirely the truth with humans, for many animals olfaction serves as the primary means by which the world around them is interpreted. Olfactory systems possess enormous sensitivity and discriminating power enabling organisms to detect and differentiate between thousands of mostly organic compounds, commonly referred to as odorants. Odors are used to locate food, enemies and mates. Insects are a notable example of a group of animals for which olfaction is of critical importance. The olfactory repertoire is represented by aliphatic and aromatic molecules with myriad classes of functional groups viz. aldehydes, ketones, esters to name a few. An understanding of the mechanisms and principles that underlie the remarkable sensory capacity of the olfactory system is a major objective for research.

In flies, as in most other insects, air borne odors are primarily detected by the antennae, which are the insects' functional equivalent of the vertebrate nose. Insect antennae come in widely differing shapes and forms, although their basic organization is similar in most if not in all insects (Keil, 1992) (Figure 2). The odor detecting part of the fly antenna is the club-shaped terminal 3rd segment, called the funiculus (Clyne et al., 1997; de Bruyne et al., 2001). In addition, flies are also capable of detecting odors with

the help of their maxillary palps (de Bruyne et al., 1999), which protrude from the mouth parts (Figure 2A). The sensory organs responsible for odor detection are the olfactory sensilla, which densely populate the fly antennae (and the maxillary palps) (Carlson, 1996; Stocker, 1994). As with the antennae, olfactory sensilla come in many different forms and shapes, however they all conform to the same principal organization (Keil,1992). A porous cuticle encloses a cavity filled with a viscous medium (the sensillum lymph) in which the dendrites of the olfactory receptor neurons (ORNs) reside. The ORNs carry the receptors that bind the odor ligands. ORNs are bipolar and the axons connect directly to the brain, in a fashion similar to that of vertebrates.

Peripheral Organization

As mentioned before, adult *Drosophila* has two principal olfactory organs: the antennae and the maxillary palp (Figure 2). The adult antenna contains on its third segment or funiculus approximately 500 sensory hairs or sensilla, which are innervated by olfactory receptor neurons (ORNs) (Figure 2). Sensilla on the surface of the funiculus can be grouped into 3 major classes (Carlson, 1996):

1. Club shaped basiconic sensilla (BS)
2. Spine shaped trichoid sensilla (TS)
3. Cone shaped coeloconic sensilla (CS)

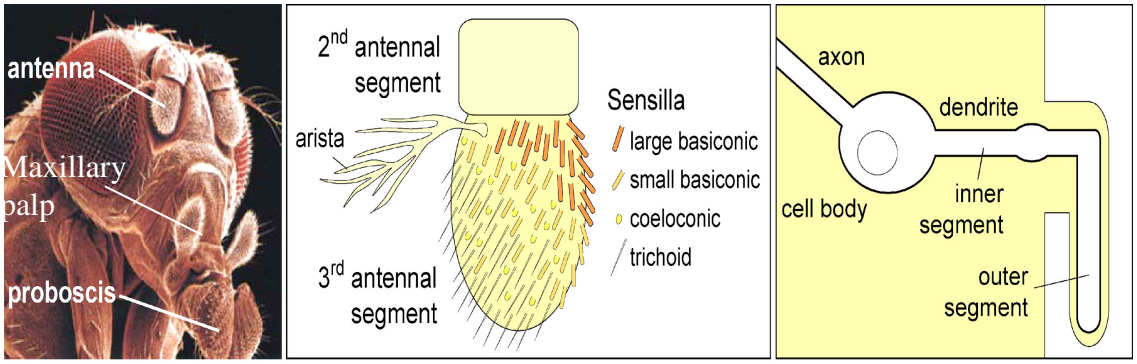

Figure 2: Organization of the *Drosophila* olfactory system. Olfactory organs – Antenna and palp are illustrated , topographic organization of sensilla, and structure of an ORN (Olfactory receptor neuron) also potrayed. (Figure from Benton et al., 2006.)

Basiconic Sensilla

Basiconics are of single walled multiporous type (Stocker, 1994). BS sensilla (~ 220 in number), are fairly wide spread in occurrence over the antennal surface. BS sensilla can be easily distinguished by light microscopy into two subtypes: large and small. The large BS are primarily seen in the basal medial surface. Large BS possesses two to four neurons, whereas the small subtype is innervated by only two neurons. Numerous pores about 30nm in diameter penetrate the wall of the sensillum and this ultra structural feature is consistent with its role in olfaction (Carlson, 1996). There are 7 different sensillar classes of BS and all are innervated by 2 neurons except for ab1, which has four ORNs (de Bryune et al., 2001).

Trichoid Sensilla

Trichoids have pores ranging up to only 10nm (Clyne et al., 1997; van der goes Naters and Carlson, 2007). Two to four neurons innervate trichoid sensilla. There are about ~ 150 sensilla trichodea on the antennal funiculus.

Coeloconic Sensilla

Coeloconics are present both on the antennal surface (~100 in number) and also in a sensory pit called the sacculus (~ 40 in number). CS are innervated by 1,2 or 3 neurons (Clyne et al., 1997; Yao et al., 2005). Sensilla of the coeloconic type have roles in both olfaction and thermoreception (Yao et al., 2005).

Maxillary Palp

The maxillary palp, which is also a mouthpart of *Drosophila*, possesses a role in olfaction (Carlson, 1996). It contains two categories of sensillum viz mechanosensory

and basiconic type sensilla. The BS number approximately 60 on the surface of the palp. It has been shown that palp deprived males exhibit higher courtship activity towards mated females than palp-intact males, suggesting a role for maxillary palp sensilla in perceiving inhibitory female compounds (Stocker et al., 1983).

Antennal Lobe

Each third antennal segment contains around 1200 olfactory neurons and each maxillary palp contains 120 neurons (Carlson, 1996). These afferent projections from the funiculus travel in the antennal nerve, and project into the antennal lobe, which is the insect counterpart of the vertebrate olfactory bulb. Also the antennal lobe is the primary olfaction association center (Stocker et al., 1983) (Figure 3). The antennal lobe also receives afferents from the maxillary palp most likely from the BS type sensilla (Stocker et al., 1983). These afferents travel through the subeosopharangeal ganglion (SOG), before innervating the antennal lobe. Like in the mammalian olfactory system, the antennal lobe is composed of anatomical subunits called glomeruli. In *Drosophila*, 43 glomeruli have been identified by morphological criteria (Laissue et al., 1999).

Afferent Projection Patterns in the Antennal Lobe

The majority of funicular ORNs (five-sixths) send axons that innervate both ipsilateral and contralateral antennal lobes. The remaining one sixth of antennal olfactory neurons project only ipsilaterally (Gao et al., 2000). In contrast, sensory neurons from the maxillary palp always project bilaterally (to both antennal lobes). Here is a clear case where the olfactory system in *Drosophila* is organized in a different way

from mammals where ORNs from each half of the nasal cavity terminate always on the same side of the olfactory bulb (ipsilateral in orientation).

Types of Neurons in the Antennal Lobes

The three types of neurons that form synapses in the antennal lobe are the Olfactory Receptor Neurons (ORN)s, projection neurons (PNs) and the local interneurons (LNs). The LN's are inhibitory in nature and the PNs form excitatory connections and serve as relay neurons between antennal lobes and higher brain centers (Stocker, 1994; Ng et al., 2002).

Lateral Horn/Mushroom Body

Information about odor coding leaves the antennal lobe via projection neurons (150-200) whose axons project to the mushroom body and lateral horn of the protocerebrum (Ng et al., 2002). The adult protocerebrum represents the anterior part of the brain and comprises highly organized neuropil structures like the mushroom bodies, the central complex and the optic lobes. Recent experiments using a genetic mosaic marking system called MARCM (Mosaic Analysis with a Repressible Cell Marker) (Lee and Luo, 1999), where individual projection neurons were labeled, show that the terminal ends of PN axons of different glomerular classes exhibit stereotypy in the axon branching pattern in the lateral horn of the protocerebrum (Wong et al., 2002).

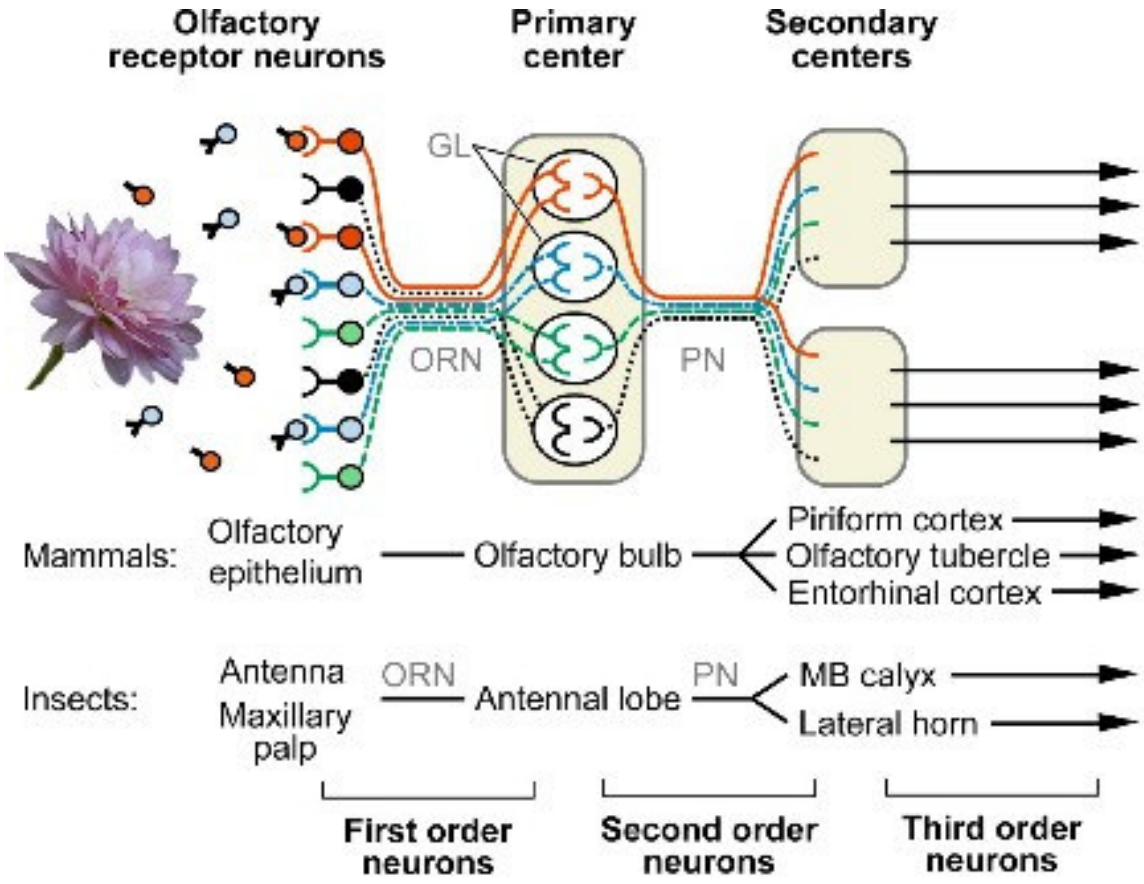

Figure 3: Processing of odor information. (Reproduced from Vosshall and Stocker, 2007.)

OLFACTORY TRANSDUCTION IN *DROSOPHILA*

Olfactory Cell Mileu

The immediate environment surrounding the olfactory organs of insects are adapted to collect odor molecules effectively and guide them to the ORN for initiating the transduction process. In *Drosophila*, odor molecules striking an antenna adsorb to the waxy surfaces of its sensilla and diffuse through narrow pores in their cuticular walls to reach the aqueous lumen inside (Carlson, 1996).

Odorant Binding Proteins

The sensillum lymph, that surrounds the dendrites of the ORNs, is an aqueous medium, a facet that causes a problem as most volatile odor ligands are hydrophobic (i.e. they dissolve poorly in water). Richard Vogt and Lynn Riddiford discovered an abundant low weight family of proteins that were exclusively located in olfactory tissue. They termed these odorant binding proteins (OBPs), and speculated that these proteins were responsible for the transportation of odor molecules through the sensillum lymph to the receptor site (Vogt et al., 1988). In *Drosophila*, OBPs are present in the fluid overlying the receptor neurons inside the sensillum. The hypothesized functions are: 1. OBPs may serve as filters before odorant stimuli arrive at the receptors. 2. OBPs form a complex with odorants before bringing it to the receptor. 3. OBPs may be involved in inactivation of odorants.

In *Drosophila* 17 OBPs have been identified so far (Robertson et al., 1999). The molecules that function as OBPs are of similar size (~14Kda), most of which are characterized by six cysteines that are found in particular parts of the sequence.

However, these molecules have diverse structures and cannot be classified as belonging to a single family (Vogt et al., 1999). LUSH is one well-characterized OBP and has been shown to bind to phthalates (Zhou et al., 2004). Interestingly, the different OBPs are expressed in different subsets of sensilla rather than broadly distributed across the antenna (Vogt et al., 1991). Recent work in *Drosophila* demonstrates the necessity of these proteins for functional olfaction. Through electrophysiological recordings from the *Lush* mutant, which lack the LUSH OBP, Dean P. Smith and coworkers managed to show that this particular OBP must be present in order to activate the receptor of the pheromone cis-vaccenyl acetate (Xu et al., 2005). This raises the possibility that OBPs could play an important role in determining which neurons respond to which odorants. Although evidently important in the olfactory pathway, the exact function of OBPs remains to be elucidated.

Odorant Receptors

Odorant receptors (ORs) are the molecules which specifically bind odorants. They belong to the superfamily of seven transmembrane domain G protein coupled receptors (GPCRs). In 1999, *Drosophila* OR's were identified by combined differential cloning (Vosshall et al., 1999).

In *Drosophila*, the ORs number at least 60 in the adult fly. The family is extremely divergent and exhibits 17 – 26% amino acid identity with each other and virtually no sequence homology to the vertebrate ORs (Vosshall, 2000). Their classification as a family relies on low sequence similarity in the seventh trans-membrane domain and their expression in olfactory systems (Firestein, 2001). Each of

the OR gene encodes a putative seven TM protein (~380 amino acids). The OR gene sequences do not contain any frame shift mutations that characterize pseudogenes which are prevalent in vertebrate genomes especially in humans. The *Drosophila* OR gene family possess discrete sub-families whose members have 40 to 60% identity. Also, in *Drosophila* each ORN expresses one conventional ligand binding OR and the atypical receptor Or83b, which is expressed in about two-thirds of all sensilla (Larsson et al., 2006). Also, Or83b is required for trafficking of receptors and functioning of bonafide OR's (Benton et al., 2006; Neuhaus et al., 2005). Another recent evidence based on both experimental and bioinformatic analysis confirms that fly OR's represent a novel family of membrane proteins with membrane topology inverted relative to that of mammalian OR's (Benton et al., 2006) suggesting that fly olfaction is modeled on an unique design principle.

Components Involved in Signaling Cascades

G Proteins

GTP binding proteins or G proteins are signal transducers which couple an activated receptor to a downstream enzyme or ion channel. These proteins are composed of three protein sub units: alpha, beta and gamma. Upon activation by binding to ligand bound receptor, the alpha subunit releases the bound GDP in exchange for GTP. The activated G protein dissociates into alpha-GTP and beta and gamma subunits. The dissociated subunits then activate downstream enzymes or ion-channels. The alpha subunit has an inbuilt GTPase, which hydrolyses the GTP and brings the subunits back together to form an inactive trimer.

In *Drosophila*, the expression of $dG_{q\alpha\text{-}3}$ is seen in the antenna and the palp (Talluri et al., 1995), suggesting the involvement of IP_3 mediated signaling pathways. Also in a heterologous expression system the injection of cDNA encoding the $G_{\alpha 15}$ subunit was sufficient to transduce signals from the DOR43a receptor to release Ca^{2+} via a PLC mediated cascade (Wetzel et al., 2001).

cAMP

In *Drosophila*, the involvement of cAMP was demonstrated by over expressing the *dunce* gene, which encodes a cAMP phosphodiestrase, specifically in the antenna leads to reduction in olfactory sensitivity behaviorally to ethyl acetate, acetone and ethanol (Martin et al., 2001). There is also strong evidence in *Drosophila* for the existence of an IP_3 based pathway In *Drosophila*, a mutant in the phospholipase C gene, norpA, have specific olfactory defects suggesting the involvement of an IP_3 based mechanism. Also, $dG_{\alpha\text{-}3,}$ an isoform of $dG_{q\alpha,}$ which is thought to activate an IP_3 dependant-signaling pathway, is expressed in chemosensory cells of the antenna. (Talluri et al., 1995). Also, heterologous expression of a *Drosophila* odorant receptor along with $G_{\alpha\text{-}15}$ subunit in Xenopus oocyte system is thought to trigger the release of Ca^{2+} through an IP_3 mediated pathway (Wetzel et al., 2001).

Adenylate Cyclase

Adenylate cyclase (AC) is a plasma membrane bound enzyme, which catalyzes the conversion of ATP to cyclic AMP. In *Drosophila*, the gene *rutabaga* codes for a Ca^{2+}/calmodulin responsive adenylate cyclase. Disruption of this gene leads to defect in olfactory learning and memory (Levin et al., 1992). However, an adenylate cyclase specific to olfactory transduction has yet to be reported.

Phosphodiestrases

Phosphodiestrases (PDEs) are molecules that hydrolyze cyclic AMP to AMP thus regulating the levels of intracellular cAMP.

In *Drosophila*, the *dunce* gene codes for a cAMP-phosphodiestrase (Davis and Kiger, 1981). Mutants of this gene show defects in olfactory learning and memory. Estimated level of the dnc-PDE was seen in the neuropils of the mushroom body.

Ion Channels

Gated channels are at the heart of odorant transduction pathways. The ability of these channels to respond to intracellular messengers and allow the passage of specific in-organic ions determines the rise time and amplitude of the olfactory receptor current and eventually the termination of the same after extinction of the stimulus.

In *Drosophila*, a cGMP-gated channel is expressed in the eyes and the antenna (Baumann et al., 1994). Though a role for this channel in olfactory transduction has been postulated, a clear structure-function relationship is still not available. Another channel involved in odorant transduction in *Drosophila* is the voltage sensitive K^+ channel called ether a go-go (*eag*), which has been shown to be involved in the transduction of a small

subset of odorants all having short aliphatic side chains (ethyl butyrate, propionic acid, 2-butanone and ethyl acetate). (Dubin et al., 1998). Also, the Ca^{2+} sensitive transient receptor potential (trp) channel in *Drosophila* has been shown to be involved in olfactory adaptation (Strortkuhl et al., 1999).

Compared to vertebrate systems, how olfactory signals are transduced in *Drosophila* is still unknown. Components of the cAMP and IP_3 signaling cascades have been identified in *Drosophila* (Baumann et al., 1994; Dubin et al., 1998). IP_3 maybe the second messenger involved, as mutants defective in various points in the signaling pathway display subtle olfactory behavioral deficits (Reisgo-Escovar et al., 1994). However, the lack of clear anosmic phenotypes in signal transduction mutants studied so far suggests that either second messenger systems could operate in *Drosophila* and essential signaling components have yet to be identified. (Vosshall, 2000).

SUMMARY AND AIMS OF THIS STUDY

Circadian oscillators are vital in synchronizing internal processes to external environmental cues. *Drosophila* presents an attractive organism for olfaction research due to the relative simplicity in organization of neural structures and powerful genetic tools available for making transgenics. However, much needs to be known in terms of organization of central and peripheral clocks and the output pathways that they govern. Also, much understanding is needed in terms of how olfactory signals are transduced in *Drosophila* and how the oscillator interacts with specific components of this cascade.

The purpose of the proposed research in this dissertation is to identify potential cellular and molecular mechanisms that mediate olfaction rhythms in *Drosophila.* To

identify cellular mechanisms, UAS-Gal4 strategies will be used for tissue specific expression of dominant negative forms of CLK (CLOCK) and CYC (CYCLE). Specifically, OR (Odorant Receptor)-GAL4 constructs will be used to achieve cell specific expression in the antenna. These methods will be used to address the degree of autonomy of peripheral oscillators from central clocks in *Drosophila*. To understand the molecular mechanisms controlling olfaction rhythms, the effect of certain molecules of the olfactory signal transduction pathway will be studied by use of respective mutants and the cell specific rescue of these proteins by UAS-Gal4 approaches. Finally, single unit recordings will be made from different basiconic sensilla in an effort to understand clock control over unitary action potentials from individual olfactory neurons. The influence of the circadian oscillator will be studied on select parameters of the single unit data obtained.

This dissertation describes experiments designed with the following specific aims in mind:

Specific Aims

1. To determine the extent of autonomy of peripheral oscillators in the antenna from central oscillators in the brain.
2. To examine potential molecular targets under clock control in the antenna.
3. To investigate how the oscillator influences single unit responses recorded from individual basiconic sensilla on the antenna.

These aims will shed light on the organization of peripheral clocks in *Drosophila.* The results of the experiments will help understand cells in the antenna

contribute to the rhythm in olfaction and the location of these cell types. Also, this project will help to uncover the point of interaction of the circadian clock with molecules of the odorant transduction pathway. The project has a broader scope in that it will impact the *Drosophila* olfaction community with new knowledge of roles of certain molecules with hitherto unknown function.

CHAPTER II

IDENTIFICATION OF CELLS THAT CONTROL OLFACTION RHYTHMS IN *DROSOPHILA* [**]

INTRODUCTION

Although the central oscillators present in small lateral ventral neurons (sLN_vs.) are not sufficient for EAG rhythms, a role for these cells cannot be eliminated. These rhythms might require oscillator function in both olfactory peripheral tissues and sLN_vs. Alternatively, EAG rhythms could be mediated solely by oscillators in one or more peripheral tissues, residing either in the antennal cells (neuronal and non-neuronal) or the antennal lobe. To tease out these possibilities, UAS-Gal4 strategies were used for tissue specific expression of dominant negative forms of core clock proteins CLK (CLOCK) and CYC (CYCLE). OR (Odorant Receptor)-Gal4 constructs were used to achieve cell specific expression of these dominant negatives in the antenna. EAG measurements were made from flies which express both the driver and the responder along with appropriate controls to study the degree of autonomy of peripheral oscillators.

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MATERIALS AND METHODS

Fly Rearing

Adult female flies are larger than males and offer the advantage of increased antennal surface were, which facilitates electrophysiological measurements. Measurements were made from flies that were less than a week old, as older flies typically develop a thickened antennal cuticle, which makes it difficult to place the recording electrode. Flies were grown on standard ‘‘cornmeal, yeast, molasses’’ media (http://fly.bio.indiana.edu/media-recipes.htm). Care was taken so as to not overcrowd the culture vial, which will reduce the size of the flies (and their antennae) due to competition for food. Typically, 5–10 flies (80% females) were placed in vial containing about 20 ml of solidified food. Flies were maintained at 25C. A day before the recordings were made, flies were transferred to a new food vial to ensure that the animals were not exposed to the strong smell of fermenting media just before recording of EAGs.

Recording Electroantennograms (EAGs)

Electrophysiological responses to odors were measured in the third antennal segment using a technique called an electroantennogram (EAG). EAGs measure the field potential produced by neuronal activity within a localized group of sensillae in response to an odor (Ayer and Carlson, 1992).

Electroantennogram Apparatus Setup

Because EAG recordings were made from a localized region on the *Drosophila* antenna (Ayer and Carlson, 1992), the recording apparatus should possess good optics to

enable reproducible electrode placement, mechanical stability to avoid vibration-related problems, and specialized electronics to amplify and record the EAG trace. The following section describes the components of the EAG recording apparatus.

Optics, Micromanipulators, and Vibration Isolation

We used an Olympus SZ 6045 dissecting microscope with 1–6.3 X objectives and an eyepiece that provides an additional 10 X magnification. The large working distance (<10 cm) allows placement of all the different manipulators in relation to the fly. Light is provided by a fiber-optic source. Any high-quality micromanipulator can be used to position electrodes. Huxley style manual micromanipulators (SD Instruments) were used to position the ground and recording electrodes. These manipulators allow both coarse and fine controlled (i.e., micrometer) movement in all three axes. Micromanipulators with the ground and recording electrodes were positioned adjacent to each other (Fig. 4). Typically, the ground electrode is positioned at a steeper angle and the recording electrode is oriented at a more oblique angle. A third coarse manipulator (Narishige, MN151) controls a glass micropipette with a long shank. This micropipette is used to lift the antenna so that the recording electrode can be placed on the anterior surface of the antenna (see under Recording Electroantennograms). A fourth coarse manipulator controls the position of the airflow tube relative to the fly (Fig. 4). This tube delivers the odor stimulus from the olfactometer (see Olfactometer Construction). Vibration isolation improves the quality and duration of recordings. Any sort of system that reduces vibration can be used; indeed, an inexpensive approach is to place a heavy marble balance table on partially inflated tire inner tubes.

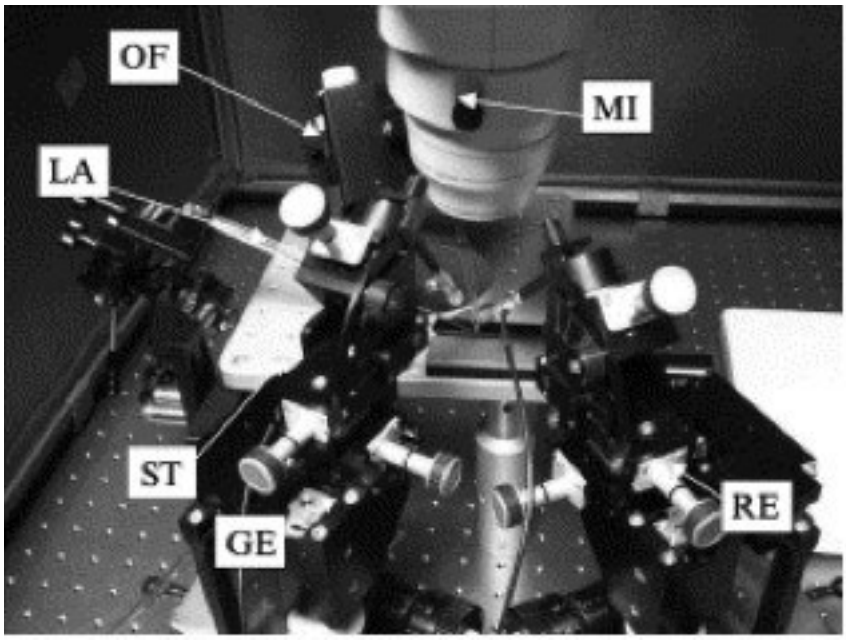

Figure 4: EAG recording apparatus. GE, manipulator that controls the ground electrode; RE, manipulator that controls the recording electrode; OF, manipulator that holds the capillary that directs airflow from the olfactometer; LA, manipulator that holds the glass micropipette used to lift the antenna; MI, dissecting microscope. (Reproduced from Krishnan et al., 2005 with permission from Elsevier.)

We use a TMC Micro-g air table that is designed for electrophysiological or optical experiments. A grounded Faraday cage is an integral component of this table, which serves to reduce electrical noise from external sources. We use a microscope platform that is screwed directly into the surface of the vibration isolation table. This stage has a moveable frame to position the fly holder that contains immobilized flies (see Fly Immobilization) relative to the recording and ground electrodes.

Electronics and Data Acquisition

Any high-quality high-impedance amplifier suitable for intracellular or extracellular recordings can be used. We used a differential amplifier (World Precision Instruments DP301), which receives input from both the recording and the ground electrode. The signal is low pass filtered at 3 kHz, and the output is passed onto a digitizer (Axon Instruments 1322A) and from there into a computer for storage and off-line analysis of data. The amplifier output is also sent to an audio monitor (Grass, AM9). The sound is low pass filtered at 300 Hz and high pass filtered at 3 kHz. This audio monitor emits a burst of high-frequency sound when the recording electrode comes in contact with the antennal surface, thus aiding electrode position in the dark—a necessity for measuring EAGs over circadian time (see Measuring EAG Responses over Circadian Time). A schematic diagram shows the arrangement of the electronic components (Fig. 5). We use Axoscope software (Axon Instruments) for data analysis. Typical EAG signals evoked by ethyl acetate were 6–14 mV in the dorsomedial region of the antenna depending on the time of day (Krishnan et al., 1999)

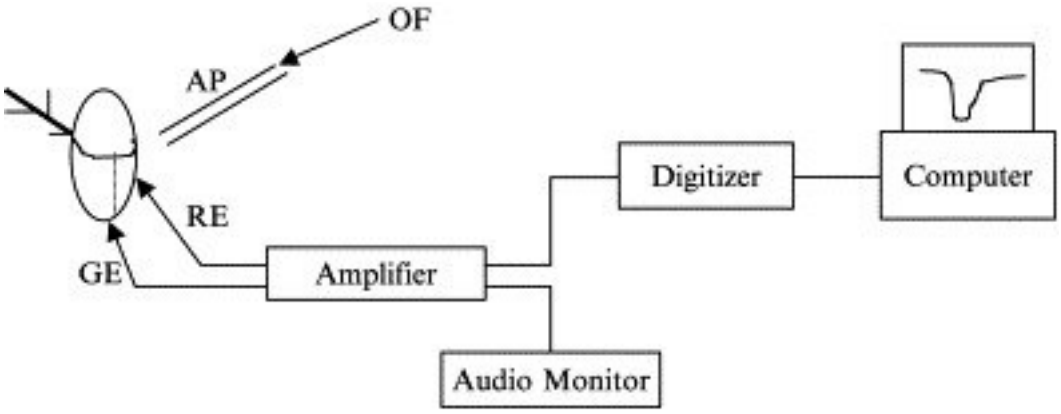

Figure 5: Electronic components of the EAG recording apparatus. An antenna is depicted on the left as an oval. The dorsomedial surface is shown, where the dashed line represents the division between the anterior and the posterior surfaces on the third segment. The arista is shown protruding from the antenna. OF, olfactometer; GE, ground electrode; RE, recording electrode; AP, port from the air pump. Arrows show the approximate positions of the GE and RE when recording circadian EAG responses to ethyl acetate. See text for further details. (Reproduced from Krishnan et al., 2005 with permission from Elsevier.)

Olfactometer Construction

To achieve controlled and repeatable stimulation of the fly preparation with the desired odorant, an olfactometer is constructed as shown in Fig. 6. Briefly, a flowmeter regulates the airflow generated by an aquarium-type aerator. From the flowmeter, a T junction separates the airflow into a vial containing 10 ml of mineral oil and into a second vial containing 10 ml of mineral containing the odorant. A second T junction receives the output from both the vials. All connections were made using Tygon tubing. Two-way valves were connected to the incoming and outgoing ports of both the vials. This allows the control of airflow from either the vial containing the mineral oil or the vial containing the odorant. The air flowing out of the second T junction is directed to the fly preparation by means of a glass tube (3-mm inner diameter). This tube is secured to a coarse manipulator to direct the airflow onto the fly preparation. The volume of odorant impinging on the fly is calculated as $\Delta V = T\, dVdt$ where ΔV is the displaced air volume, T is the duration of valve opening, and dV/dt is the airflow rate. Typically, the volume ranges from 10 to 15 ml of the odorant in the vapor phase. Note that with this system, the fly is always exposed to flowing air. What changes is whether the air contains odorants. This is essential to prevent mechanical stimulation from contributing to the EAG signal.

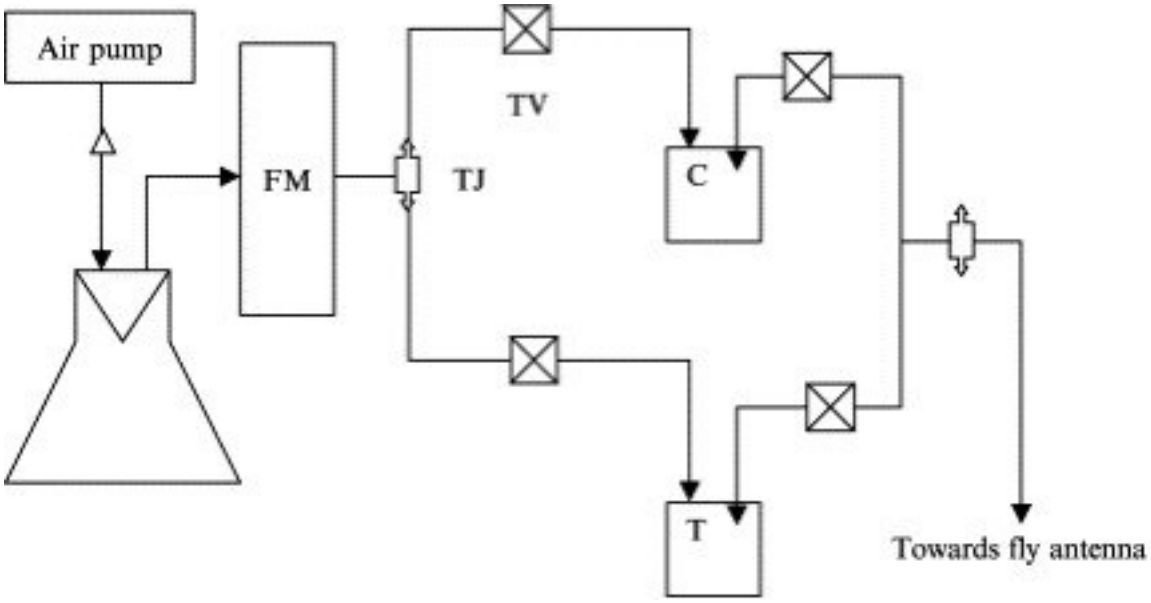

Figure 6: Schematic diagram of an olfactometer. FM, flowmeter; TV, two-way valves; C, vial containing mineral oil; T, vial containing the odorant diluted in mineral oil; TJ, T junction. See text for further details. (Reproduced from Krishnan et al., 2005 with permission from Elsevier.)

Housing the EAG Apparatus

The room in which the apparatus is housed is provided with a HEPA filter-based air purifier and is located in an area of the laboratory away from experimental stations using strong odoriferous chemicals such as acetone, methanol, and acetic acid. The room was also made completely dark to facilitate circadian measurements (see Measuring EAG Responses over Circadian Time).

Preparing to Record EAG Responses

Once the equipment is in place for recording EAG responses, animals and consumables was assembled. Animals produced for EAG measurements were large, healthy, and unaffected by external odor sources before they were immobilized for recording. Likewise, odorants and electrodes must be freshly prepared to ensure that EAG response amplitudes were robust and reproducible. This section describes the rearing and immobilization of animals, as well as the preparation of odorants and electrodes for EAG recordings.

Immobilizing Flies

Flies were transferred to an empty glass vial, which is immersed in a bucket of ice for about a minute to produce anesthesia. The anaesthetized flies were placed on a Whatman filter paper on top of an aluminum block, which is cooled from below by ice. The flies were then gently picked up by the wings using forceps and were placed on a ‘‘L’’-shaped plexiglass ‘‘fly holder’’ (Fig. 7), which rests on top of the aluminum block. This fly holder has 1-mm-diameter holes drilled every 10 mm along its length on the wide portion of the ‘‘L.’’ A fly is inserted into each hole such that the head protrudes out

and the rest of the body lies flat, with the wings against the surface of the short portion of the ‘‘L’’ (Fig. 4). A small amount of lowmelting- point wax (myristic acid, 58.5 melting point) is melted using a fine silver wire heated by an adjustable power source of 10–15 V (Staco Energy, 3PN1010V). The wax is melted just until it can be spread, and care is taken not to overheat the wax. This wax is then gently applied to opposite sides of the head, making sure that the silver wire never comes into contact with the fly preparation, but is sufficiently close that the wax spreads by capillary action alone. This effectively glues the fly to the fly holder. The wings and the legs were also immobilized with melted wax. Three to five animals were fixed in a single fly holder spaced in such a way that there is an empty hole between two flies. This is done to prevent the odor stimulus applied to one fly from affecting the neighboring fly. This immobilization procedure is done as quickly as possible to avoid dehydration of flies. With practice, about 10 flies can be immobilized in 15–20 minutes. After fixing the flies, the fly holder is moved to a moist chamber, which is composed of a 14-cm petri dish lined with a sheet of moistened Whatman filter paper. The immobilized flies remain in this chamber for up to 1 h before EAG recordings were made.

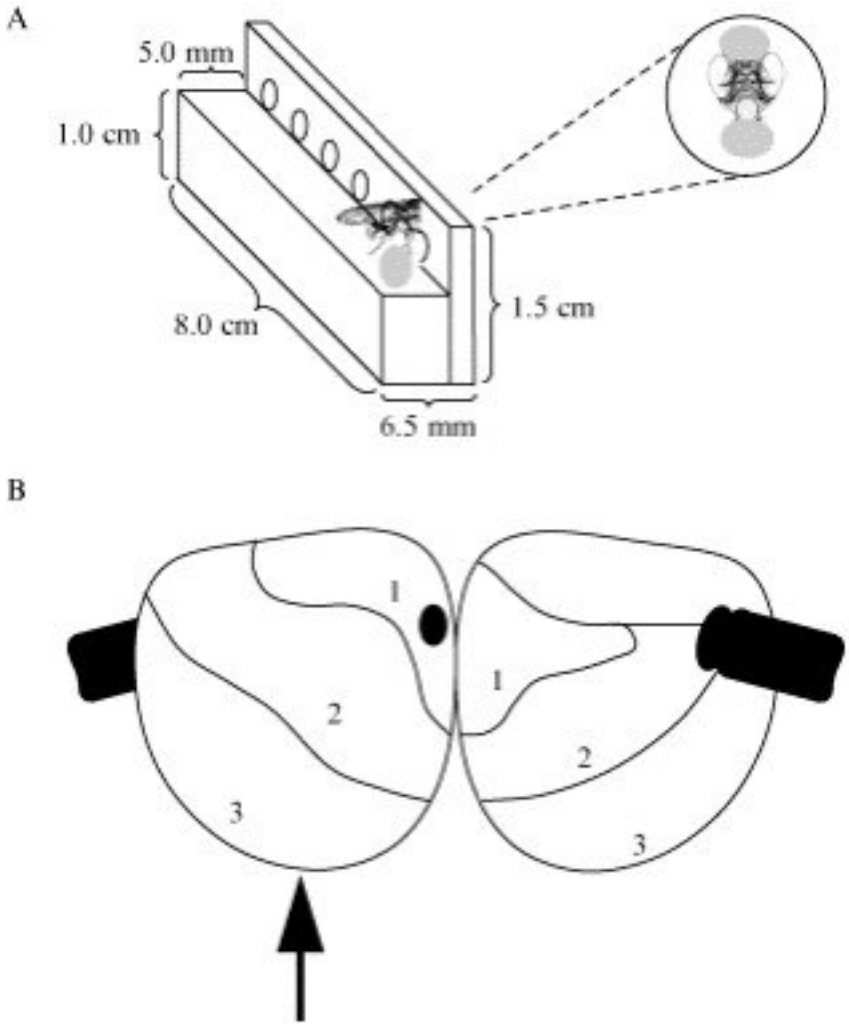

Figure 7: Fly immobilization and electrode placement. (A) Side view of the fly holder showing an immobilized fly in the last position. The top view shows the head of the immobilized fly protruding from the hole. Wax placed on opposite sides of the head and on the fly body is depicted as gray ovals. (B) Diagram of a fly antenna showing regions rich in different types of sensillae. Left side, anterior face; right side, posterior face; top, dorsal; bottom, ventral. 1 denotes the subregion rich in large basiconic sensillae, 2 denotes the subregion rich in small basiconic sensillae, and 3 denotes the subregion rich in trichoid sensillae. Coeloconic sensillae are present at low density in subregions 2 and 3. The black oval denotes the site from which recordings to ethyl acetate. The arrow denotes where the ground electrode penetrates the antenna. The base of the arista (black shape) is shown protruding from the antenna. The diagram is based on de Bryune et al., 2001. (Reproduced from Krishnan et al., 2005 with permission from Elsevier.)

Fabrication of Micropipettes

Pipettes for EAG recording were made from borosilicate glass capillary tubes (Drummond #2-200-210). Capillaries were pulled to an internal tip diameter of 1–2 _m using a two-stage vertical micropipette puller (Narshige, PP830). Other electrode pullers capable of pulling patch clamp microelectrodes would also be suitable. The pipettes were back-filled with 0.17 M NaCl solution. Bubbles near the tip were removed by tapping the pipette gently. The pulled pipettes can be used up to 2 h after fabrication. A glass micropipette used for lifting the antenna is made from thin capillary tubes (Drummond #1-000-0500 Microcaps) pulled using a vertical one-stage puller (Kopf 720).

Preparation of Odor Dilutions

Odorants were diluted in light mineral oil. Most of our studies have focused on ethyl acetate, which is serially diluted starting from 1 ml of neat ethyl acetate and 9 ml of mineral oil (both from Sigma). After vortexing, the same procedure is repeated three times to reach a dilution of 10^{-4} that is used for EAG measurements. Each dilution is vortexed for 20 s to ensure thorough miscibility of the odorant in mineral oil. Glass pipettes or one-time-use disposable pipettes were used to make serial dilutions of the odorant, and a fresh dilution of the odorant is made just before each recording session. Care was taken to ensure that the vials were airtight and sealed properly.

Chloridization of Electrodes

The silver electrode wires of both ground and recording electrodes were immersed in concentrated bleach for about 15 min until the surface of the wires were oxidized and have a dark brown or dull gray appearance. Chloridization by this method ensures a stable baseline in the EAG trace. Care should be taken while inserting the filled micropipettes into the holder so as to not scratch the surface of the chloridized silver wire.

Recording Electroantennograms

EAG recordings were done at a temperature of 25C with a relative humidity of 50–80%. Recording is possible from various sites on the third antennal segment that were rich in different sensillae classes (Stocker, 2001). The antenna is lifted to expose a high density of basiconic sensillae on the anteromedial face (Krishnan et al., 1999). The following describes the steps used in recording an EAG. An electroantennogram trace in response to ethyl acetate is shown is shown in Figure 8.

Positioning the Electrodes

Because the recording is done from the anterior region of the antenna, which is not immediately accessible, the left antenna is gently lifted with the help of a micropipette with a very long shank length. The dorsomedial region of the anterior surface of the antenna contains primarily basiconic sensilla, many of which respond robustly to ethyl acetate (Ayer and Carlson, 1992; de Bruyne et al., 2001). The antenna is lifted such that the dorsomedial surface is facing the manipulator with the recording

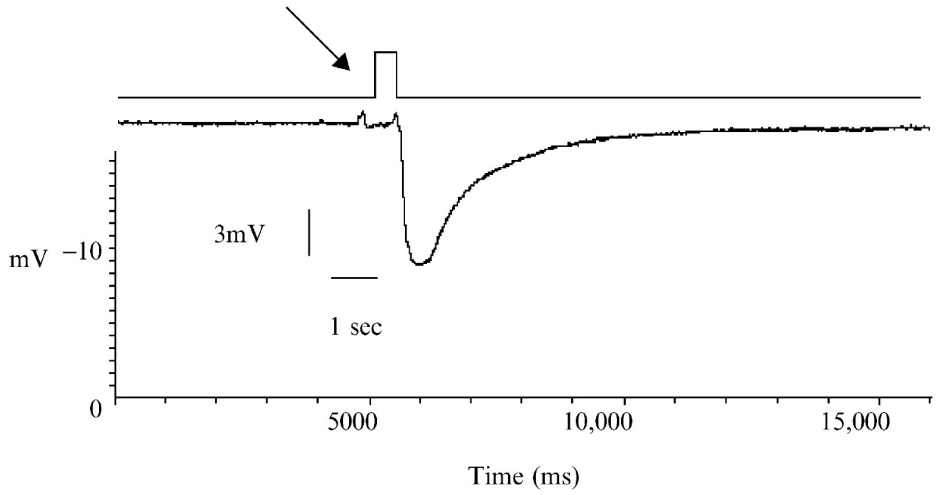

Figure 8: An electroantennogram (EAG) trace in response to an odor stimulus. Arrow indicates application of a ~1 second pulse of 10^{-4} dilution of ethyl acetate. Response recorded from wildtype (CS) flies at ZT 5. (Reproduced from Krishnan et al., 2005 with permission from Elsevier.)

electrode (Fig. 9). The manipulator with the ground electrode is advanced until the tip of the electrode is in contact with the distal tip of the antenna. The manipulator is then tapped gently so that the electrode tip is inserted into the lumen of the antenna. Once the ground electrode is in place, the micropipette used to lift the antenna is gently moved away and the antenna is supported solely by the ground electrode in the lumen.
The recording electrode is then placed onto the dorsomedial surface of the third antennal segment such that the tip just impinges on the surface of the antenna.

Stimulus Delivery and Response

The airflow tube from the olfactometer is placed about 5 mm from the fly preparation. The Axoscope program is started, and an active oscilloscope window is opened and small changes in electrode positions were made until a stable baseline is achieved. After obtaining a stable baseline reading, the baseline is adjusted to zero using the DC shift knob on the amplifier. There is a constant flow of air on the preparation through the olfactometer, which can deliver odor stimuli by opening and closing of the two-way valve connected to the vial containing the odorant with the simultaneous closing and opening of the valve connected to the vial with the mineral oil. Odorant delivery evokes a change in potential, which is stored for analysis. The traces for individual flies were highly reproducible (Krishnan et al., 1999), thus only one trace is recorded from each fly. Recordings were made from at least eight independent flies, and the resulting amplitude values in millivolts (mVs) were used to compute the mean and standard error of EAG responses. Note that with sustained delivery of odorant, the

responses desensitize. Responses to ethyl acetate typically recover from desensitization in 30 s (Krishnan et al., 1999).

Measuring EAG Responses Over Circadian Time

Adult female flies were entrained in incubators set to 12-h light:12-h dark (LD) cycles for 4 days. Time during LD conditions is referred to as Zeitgeber time (ZT), where ZT0 is defined as lights on and ZT12 is defined as lights off. Measurements made every 4 hours during an LD cycle were sufficient to reliably detect a rhythm. EAG responses measured during the light phase (i.e., ZT1, ZT5, and ZT9) were done using the protocol described earlier for recording EAGs. Typically the EAG amplitude during the light phase is close to the trough level of 8 mV (To record responses during the dark phase (i.e., ZT13, ZT17, and ZT21), flies were transferred from the incubators to the EAG room in a ‘‘light-tight’’ box that is typically used to transport negative photo-films prior to developing them. Flies were insensitive to wavelengths of light above 600 nm (Klemm et al., 1976; Suri et al., 1998). All light sources in the EAG room were shielded with filters that emitted light in the near-infrared region (>600 nm). These filters were red translucent cellophane sheets that were spectrophotometrically tested to transmit light above 600 nm. In addition, walls of the EAG room were painted black to ensure that flies were not affected by any incidental light source. Recordings conducted during the dark were tedious at first, but with practice they become more manageable. EAG responses during the dark phase increase to a peak of 14 mV at ZT17 and then decrease to trough levels as lights turn on. Recordings can also be made during constant dark (DD) conditions. Time during DD conditions is referred to as circadian time (CT), where

CT0 is defined as the time when lights would have turned on (i.e., subjective lights on) and CT12 is defined as the time when lights would have turned off (i.e., subjective lights off). To record EAG responses under these conditions, flies were first entrained to LD cycles for 4 days as described earlier. Flies were then transferred to an incubator set to constant darkness and collected every 4 h (i.e., CT1, CT5, CT9, CT13, CT17, and CT21). Flies were transferred to the EAG room in a light-tight box, immobilized, and recorded as described previously. Under DD conditions, the phase and amplitude of the EAG rhythm were similar to that seen under LD conditions (Krishnan et al., 1999, 2001; Tanoue et al., 2004).

Gal4 Drivers and UAS Responders Used for Cell Specific Expression

Gal4 drivers used for this experiment are as follows: pdf-Gal4 (expressed only in the lateral neurons), Or83b-Gal4 (expressed in almost all antennal sensory neurons), 22a-Gal4(expressed only in the ab3 sensilla class), tim-Gal4 (for expression in all oscillator cells). The UAS responders used will be UAS-CLKΔ and UAS-CYCΔ to express the dominant negative forms of CLK and CYC respectively.

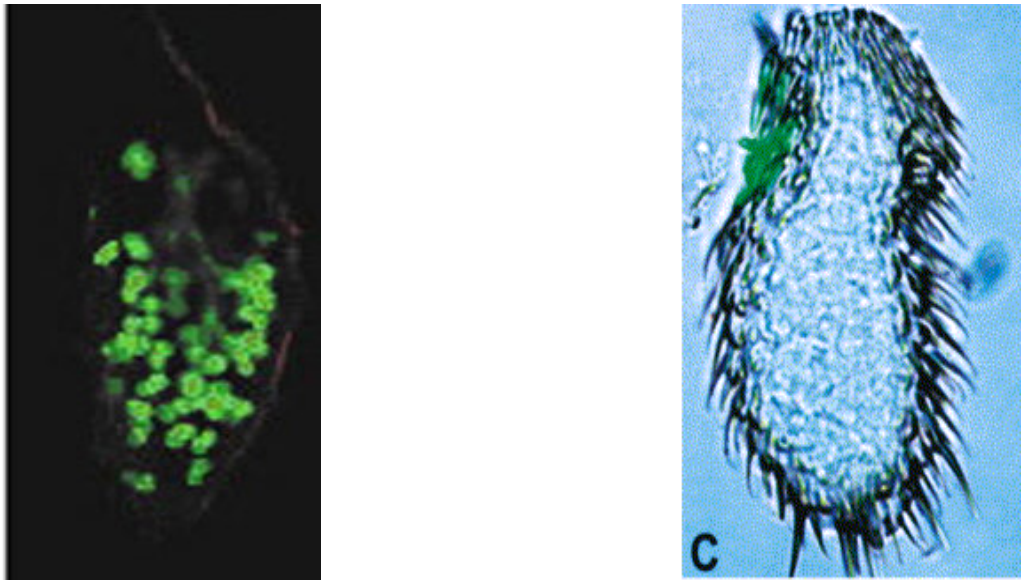

Figure 9: Gal4 drivers used for tissue specific expression. Or83b expression profile in about 2/3rds of all sensilla (right). Or22a expression pattern in only the ab3 sensilla (left). (Figures from Larsson et al., 2005 and Dobritsa et al., 2003 respectively.)

Working Model of Dominant Negatives of CLK and CYC

Cell ablation by expressing apoptotic genes in the antenna would most likely result in the loss of olfactory function in the antenna i.e. no EAG responses to odorants. To avoid this potential problem, Dr. Shintaro Tanoue , designed dominant-negative versions of CLK and CYC so that clock function could be eliminated without affecting the ability of these cells to respond to odors. CLK and CYC have three common functional domains: a basic region that is responsible for DNA binding, an HLH that mediates dimerization of bHLH factors, and a PAS domain that is responsible for interactions with other proteins. To generate CLK and CYC dominant-negatives, a portion of their basic regions were removed to impair DNA binding while retaining the ability to form heterodimers. This modified structure is similar to that of the natural dominant-negative ID proteins, which block the ability of certain bHLH factors to activate transcription. A total of 15 and 17 amino acids were removed from the CLK and CYC basic regions, respectively, and the resulting proteins, CLKΔ and CYCΔ, were tested for their ability to block CLK and CYC function in vitro and in vivo. Below is a working model of dominant negative versions of CLK.

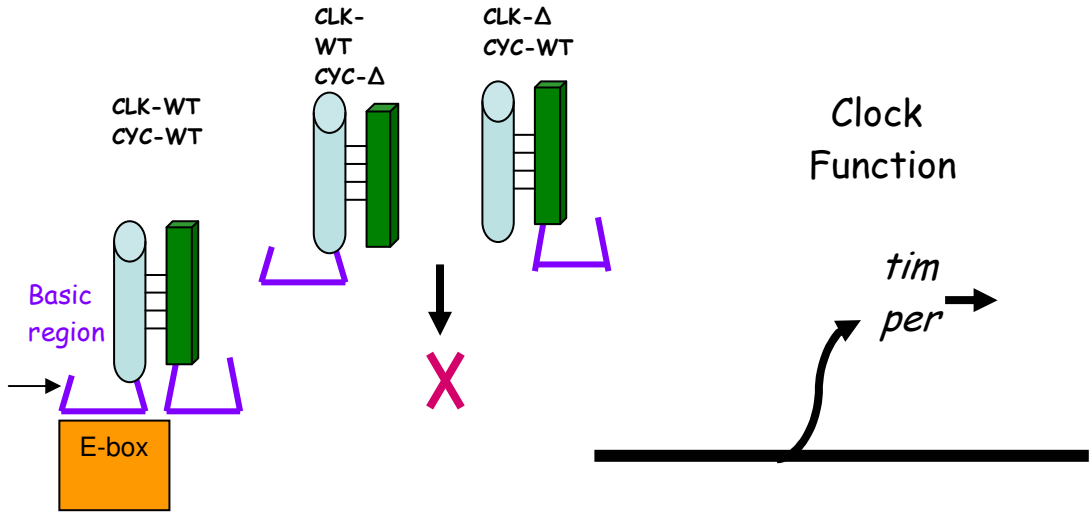

Figure 10: Working model of dominant negative forms of CLK and CYC.

RESULTS

PDF Output Is Not Necessary for Sustaining Rhythms in Olfaction

To determine whether LN_vs , which are both necessary and sufficient for locomotor activity rhythms, are also required for EAG rhythms, ablation of these cells was achieved using the LN_v-specific *pdf* promoter to drive the cell death genes *reaper* (*rpr*) and *head involution defective* (*hid*) by using the GAL4/UAS system as described previously (Renn et al., 1999).These experiments were performed by Dr.Balaji Krishnan. Given that a single LN_v can support rhythmic locomotor activity (Helfrish-Foster et al., 1998 and 2000) and the lack of behavioral rhythms in flies containing both transgenes indicates that the LN_vs were effectively ablated. EAG responses were measured to ethyl acetate on the second day of DD in flies containing three independent *pdf*-Gal4 inserts with or without the UAS-*rpr*, *hid* inserts, and in UAS-*rpr*, *hid* alone and found that the responses of flies that lack LN_vs were similar to those in control strains (i.e., strains that contain the Gal4 driver or the UAS responder transgenes alone) which have unperturbed LN_vs . This result indicates that output from LN_vs are not necessary for EAG rhythms. These results are illustrated in Figure 11.

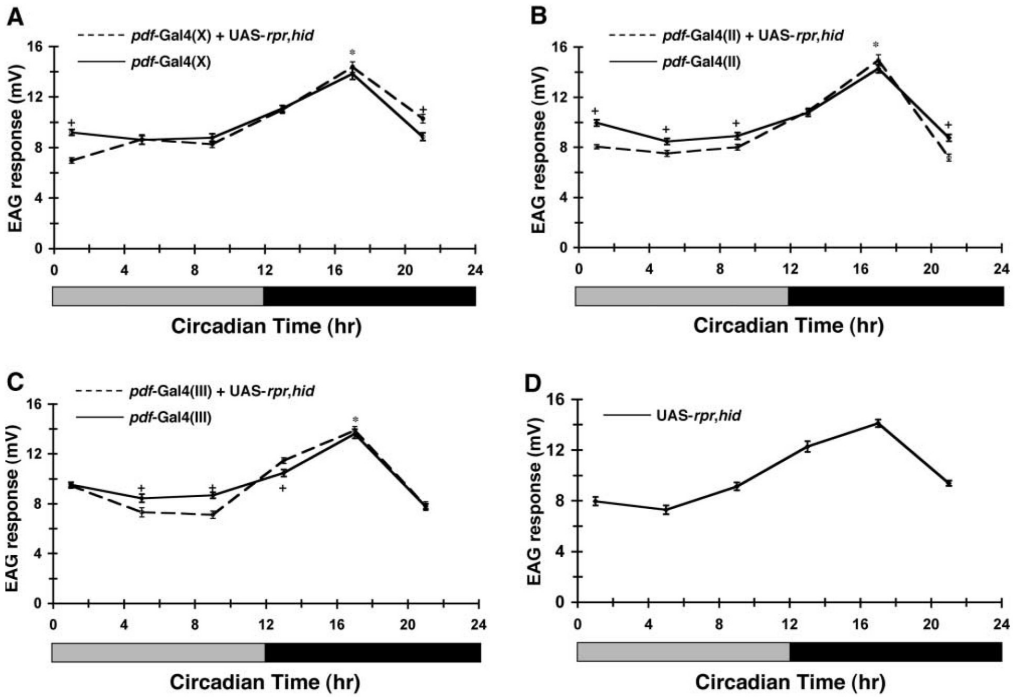

Figure 11: EAG responses are rhythmic in flies lacking LN_vs. Each point represents mean odor-evoked EAG responses in the specified strain. Circadian changes in mean EAG responses are plotted for ethyl acetate on day 2 of DD. Each point represents the mean of at least 24 females. The gray bar indicates subjective day, and the black bar denotes subjective night. Asterisks denote a significant ($p < 0.05$) increase in EAG responses in *pdf*-Gal4 control flies at ZT17 compared to other times of day. Crosses denote significant ($p < 0.01$) differences in EAG responses between the LN, ablated genotypes at the same circadian time. Error bars denote SEM. (A) EAG responses of flies containing an X chromosome *pdf*-Gal4 insert with or without UAS-*rpr,hid*. (B) EAG responses of flies containing an second chromosome *pdf*-Gal4 insert with or without UAS-*rpr,hid*.(C) EAG responses of flies containing a third chromosome *pdf*-Gal4 insert with or without UAS-*rpr,hid*. (D) EAG responses of flies containing the UAS-*rpr,hid* insert. (Reproduced from Tanoue et al., 2004, with permission from Elsevier.)

CLK and CYC Dominant-Negatives Abolish EAG Rhythms

To determine if CLKΔ and CYCΔ can block wild type function of these proteins in vivo, we generated flies containing the UAS-CLKΔ and UAS-CYCΔ transgenes. A *tim*-Gal4 driver was used to drive expression of these dominant-negatives universally in all clock cells (Kaneko and Hall, 2000). EAG measurements of flies which had both the *tim*-Gal4 driver with either the UAS-CLKΔ or UAS-CYCΔ responders had olfaction rhythms eliminated while control strains with either the driver and responder alone showed persistence of olfaction rhythms (Figure 12).

Clocks in Antennal Neurons Are Necessary for EAG Rhythms

Since dominant negative versions of CLK and CYC abolish oscillator function in-vivo, these constructs can be used to investigate which oscillator cells in the antenna are necessary to maintain olfaction rhythms. Odorant receptor (Or) promoter-regulated Gal4 drivers were used because they are expressed specifically in subsets of olfactory receptor neurons (ORNs) which are the cells that produce the EAG response (Ayer and Carlson, 1992). Since basiconic sensillae show robust rhythms to ethyl acetate (de Bryune et al., 2001; Dobritsa et al., 2003). EAG responses in flies expressing CLKΔ and CYCΔ in antennal neurons were recorded from flies maintained in LD cycles. Under these conditions, robust EAG rhythms are seen in wild-type flies, but abolished in mutants that eliminate clock function (Krishnan et al., 1999; Krishnan et al., 2001). The most widely expressed OR promoter, OR83b was used first to drive CLKΔ and CYCΔ expression in 2/3rds of all antennal ORNs.

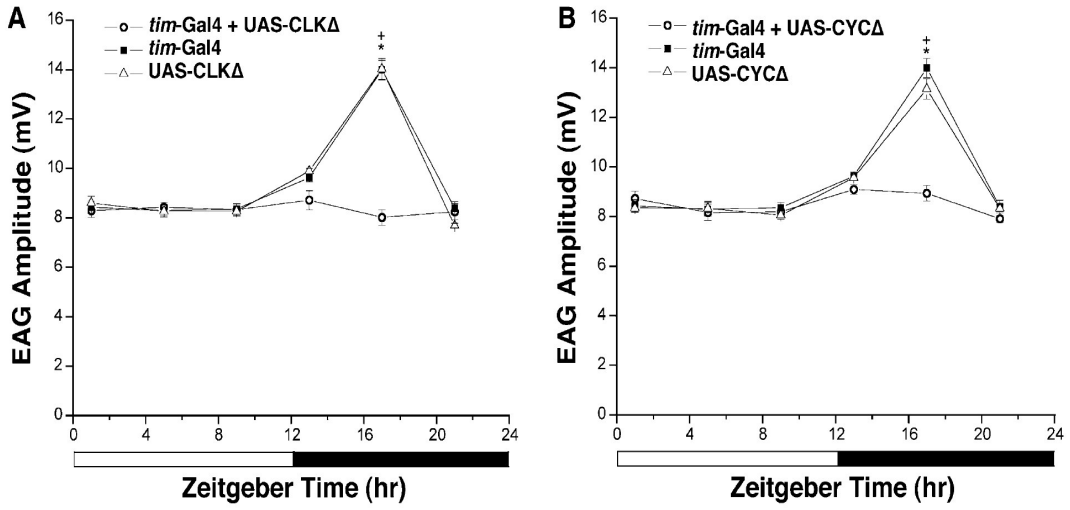

Figure 12: *tim* promoter-mediated CLKΔ and CYCΔ expression abolishes EAG rhythms. Diurnal changes in mean EAG responses are plotted for ethyl acetate on day 4 of LD. Each point represents the mean of at least eight females. The white and black bars indicate times when lights were on and off, respectively. Asterisks denote significant ($p < 0.01$) increases in EAG responses for flies carrying *tim*-Gal4, UAS-CLKΔ, or UAS-CYCΔ transgenes at ZT17 compared to other times of day. Crosses denote significant ($p < 0.01$) differences in EAG responses between flies carrying *tim*-Gal4, UAS-CLKΔ or UAS-CYCΔ transgenes and *tim*-Gal4 + UAS-CLKΔ or *tim*-Gal4 + UAS-CYCΔ transgenes at the same time of day. Error bars denote SEM.(A) EAG responses of flies containing *tim*-Gal4, UAS-CLKΔ, or *tim*-Gal4 + UAS-CLKΔ. (B) EAG responses of flies containing *tim*-Gal4, UAS-CYCΔ or *tim*-Gal4 + UAS-CYCΔ. (Reproduced from Tanoue et al., 2004, with permission from Elsevier.)

Expression of either CLKΔ or CYCΔ in Or83b-expressing ORNs abolished EAG rhythms, while control flies containing the Or83b-Gal4 driver, UAS-CLKΔ responder, or UAS-CYCΔ responder transgenes alone showed wild-type rhythms (Figure 13). This result shows that circadian oscillators in Or83b-expressing ORNs are required for EAG responses. To further define oscillator neurons that are necessary for EAG rhythms in response to ethyl acetate, we used Or22a-Gal4 to drive dominant negatives of CLK and CYC expression specifically in about 30 basiconic sensillae of the ab3 subtype. Flies expressing either CLKΔ or CYCΔ in Or22a-expressing ORNs show no rhythms in EAG responses (Figure 14). Given that the ab3 class of large basiconic sensillae is restricted to the dorsomedial region of the antenna (Dobritsa et al., 2003), we reasoned that placing the recording electrode in a more lateral region which lack the ab3 class of sensilla, would continue to exhibit EAG rhythms in flies expressing a CLK or CYC dominant-negative in Or22a-expressing ab3 cells. When flies expressing CYCΔ in Or22a cells were recorded from a more lateral position, EAG responses were indeed rhythmic, although at a lower amplitude (Figure 14). Taken together, these results demonstrate that oscillators contained in the antennal neurons are necessary for rhythms in EAG responses.

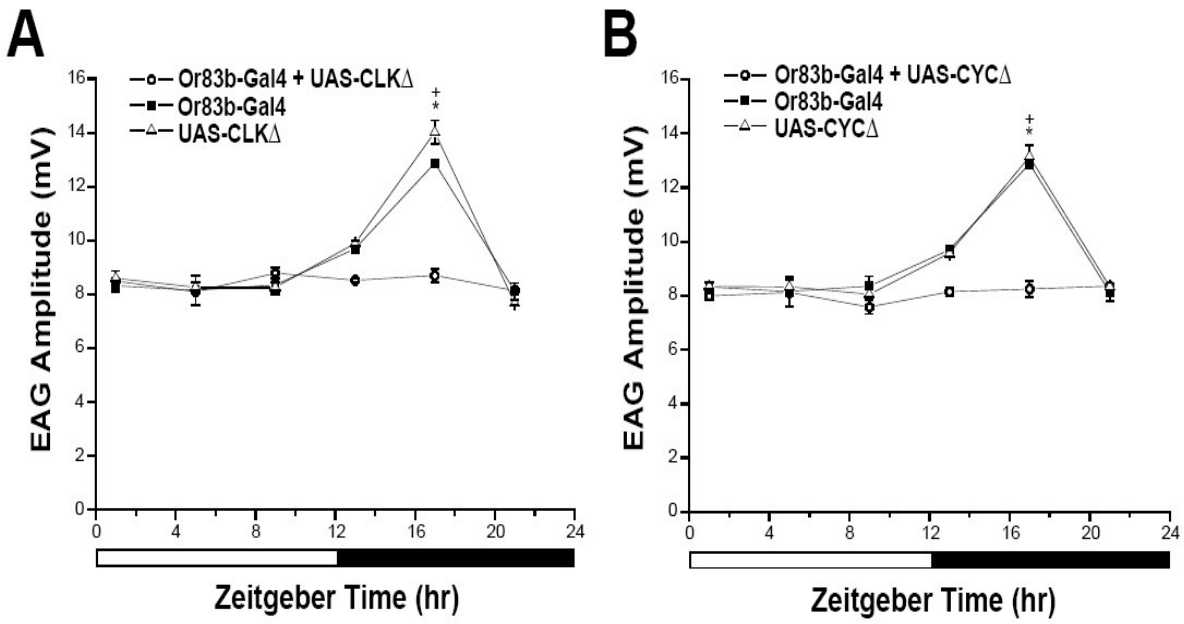

Figure 13: Or83b-mediated CLKΔ and CYCΔ expression abolishes EAG rhythms. EAG recordings were performed as described in the Methods section. Asterisks denote significant ($p < 0.01$) increases in EAG responses for flies carrying Or83b-Gal4, UAS-CLKΔ, and UAS-CYCΔ transgenes at ZT17 compared to other times of day. Crosses denote significant ($p < 0.01$) differences in EAG responses between flies carrying Or83b-Gal4, UAS-CLKΔ, or UAS-CYCΔ transgenes and OR83b-Gal4 + UAS-CLKΔ or OR83b-Gal4 + UAS-CYCΔ transgenes at the same time of day. Error bars denote SEM. (A) EAG responses of flies containing Or83b-Gal4, UAS-CLKΔ, or OR83b-Gal4 + UAS-CLKΔ. (B) EAG responses of flies containing Or83b-Gal4, UAS-CYCΔ, or OR83b-Gal4 + UAS-CYCΔ. OR83b-Gal4 + UAS-CYCΔ. (Reproduced from Tanoue et al., 2004, with permission from Elsevier.)

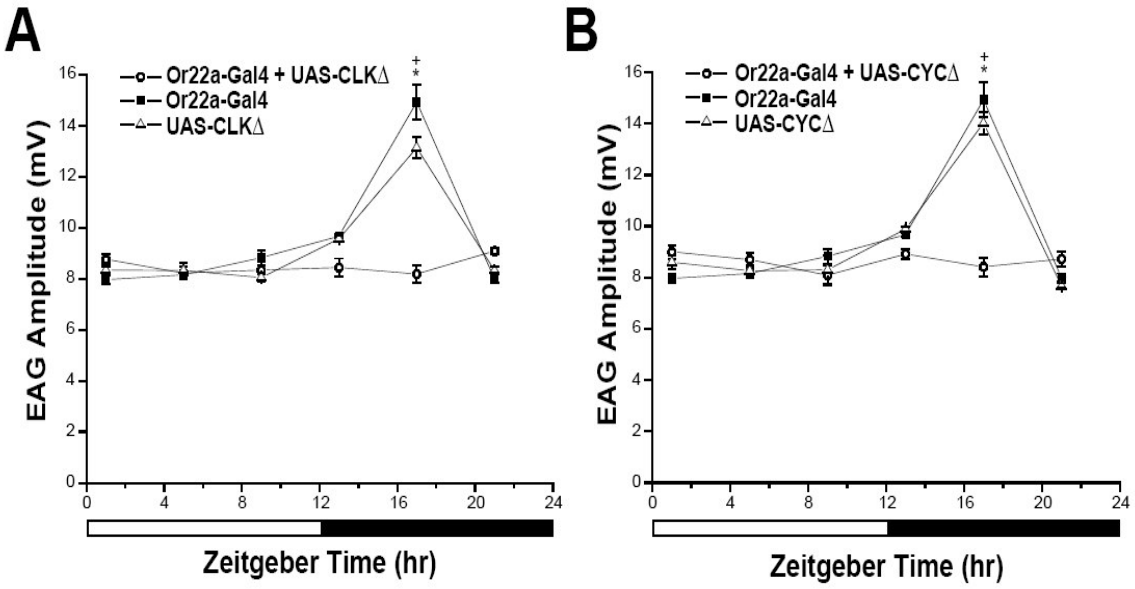

Figure 14: Or22a-mediated CLKΔ and CYCΔ expression abolishes EAG rhythms. EAG recordings were performed as described earlier. Asterisks denote significant ($p < 0.01$) increases in EAG responses for flies carrying Or22a-Gal4, UAS-CLKΔ, and UAS-CYCΔ transgenes at ZT17 compared to other times of day. Crosses denote significant ($p < 0.01$) differences in EAG responses between flies carrying Or22a-Gal4, UAS-CLKΔ, or UAS-CYCΔ transgenes and OR22a-Gal4 + UAS-CLKΔ or OR22a-Gal4 + UAS-CYCΔ transgenes at the same time of day. Error bars denote SEM. (A) EAG responses of flies containing Or22a-Gal4, UAS-CLKΔ, or OR22a-Gal4 + UAS-CLKΔ. (B) EAG responses of flies containing Or22a-Gal4, UAS-CYCΔ, or OR22a-Gal4 + UAS-CYCΔ. (Reproduced from Tanoue et al., 2004, with permission from Elsevier.)

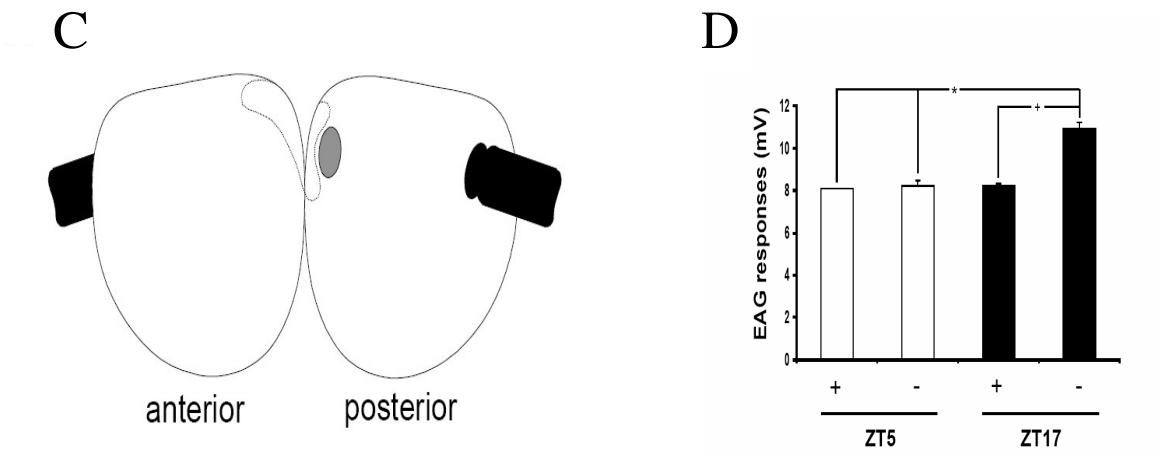

Figure 14: (contd) (C) Diagram of the anterior and posterior faces of the antenna denoting the region where Or22a is expressed (dashed outline). Recording electrodes were positioned within Or22a-expressing cells or in a more lateral position that contains other ethyl acetate responsive large basiconic sensillae (shaded area). The antenna is oriented dorsal side up, and the black shapes represent the aristae.
(D) EAG responses within and outside of a region in which Or22a neurons are expressing CYCΔ. EAG recordings were performed as described before at ZT5 and ZT17 in the two antennal regions shown in C. The plus symbol indicates the region in which Or22a is expressing CYCΔ, and the minus symbol indicates the lateral region, which lacks Or22a neurons expressing CYCΔ. The white bars represent EAG responses measured during the light phase at ZT5, and the black bars represent EAG responses taken during the dark phase at ZT17. The cross denotes a significant ($p < 0.00001$) difference in EAG responses between the region in which Or22a neurons are expressing CYCΔ and the region that lacks Or22a neurons expressing CYCΔ at ZT17. The asterisk denotes significant ($p < 0.00001$) differences in EAG responses between the lateral region that lacks Or22a neurons expressing CYCΔ at ZT17 and either antennal region at ZT5. Error bars denote SEM. (Reproduced from Tanoue et al., 2004, with permission from Elsevier.)

Oscillators in Antennal Neurons Are Sufficient for EAG Rhythms

Since central oscillators in the lateral neurons are neither necessary nor sufficient for olfaction rhythms, it is possible that a peripheral oscillator in the antenna which is necessary for EAG rhythms is also sufficient to maintain these rhythms. To determine if this is the case, it is necessary to generate flies that have functional circadian oscillators only in antennal neurons. This was attained by rescuing the cyc^{01} phenotype with a wild type version of CYC in antennal neurons via targeted expression of the *cyc* gene. Since targeted expression in antennal neurons by using *Or-Gal4* drivers would produce constant levels of target transcripts, we have used these promoters to express *cyc*, since it is the only constitutively expressed transcriptional regulator in the core clock mechanism (Rutila et al., 1998). Importantly, previous work has shown that targeted *cyc* expression can rescue circadian oscillator function in cyc^{01} flies (Peng, 2003). The Or83b-Gal4 and UAS-*cyc* transgenes were introduced into cyc^{01} mutant flies, and these two strains were crossed to produce flies that express CYC in most antennal neurons. EAG responses in cyc^{01} flies containing the Or83b-Gal4 and UAS-CYC inserts were rhythmic, whereas cyc^{01} flies containing either insert alone were arrhythmic (Figure 15).

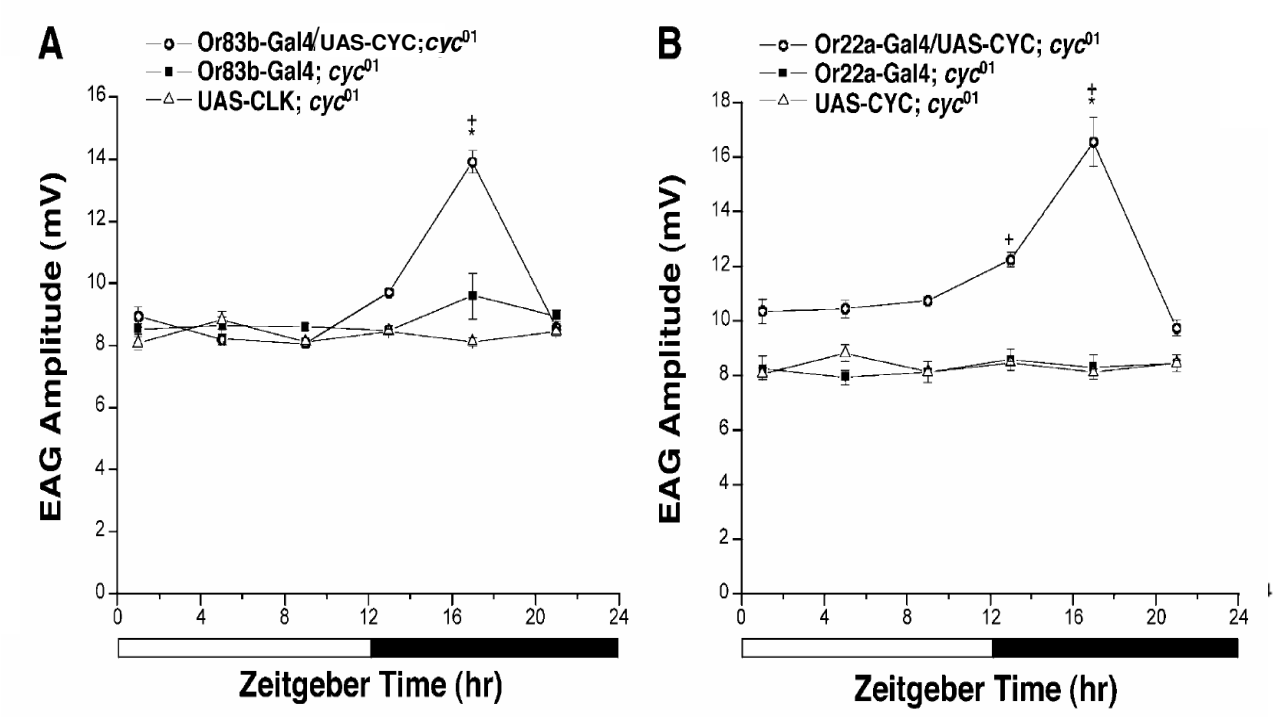

Figure 15: Or83b and Or22a-mediated CYC expression restores EAG rhythms in cyc^{01} flies. EAG recordings were performed as in described before**.** Asterisks denote significant ($p < 0.01$) increases in EAG responses for cyc^{01} flies carrying Or83b-Gal4 + UAS-CYC or Or22a-Gal4 + UAS-CYC at ZT17 compared to other times of day. Crosses denote significant ($p < 0.01$) differences in EAG responses between cyc^{01} flies carrying Or83b-Gal4 + UAS-CYC or Or22a-Gal4 + UAS-CYC transgenes and cyc^{01} flies carrying Or83b-Gal4, Or22a-Gal4, or UAS-CYC transgenes at the same time of day. Error bars denote SEM.(A) EAG responses of cyc^{01} flies carrying Or83b-Gal4, UAS-CYC, or Or83b-Gal4 + UAS-CYC. (B) EAG responses of cyc^{01} flies carrying Or22a-Gal4, UAS-CYC, or Or22a-Gal4 + UAS-CYC. Overall effects of time of day, genotype, and their interaction are statistically significant ($p < 0.00001$) by two-way ANOVA. (Reproduced from Tanoue et al., 2004, with permission from Elsevier.)

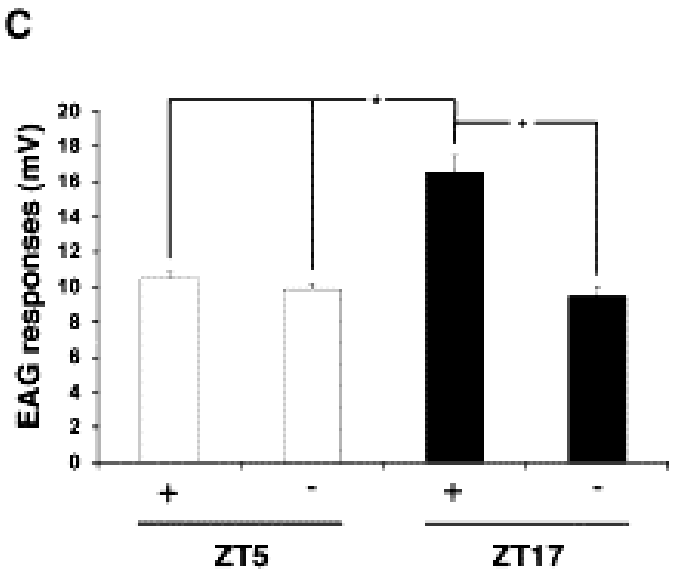

Figure 15 (Contd.): (C) EAG responses within and outside of a region in which Or22a neurons are expressing CYC in *cyc*^{01} flies. EAG recordings were performed at ZT5 and ZT17 in the two antennal regions shown in Figure 15. The plus symbol indicates the region in which Or22a is expressing CYC in *cyc*^{01} flies, and the minus symbol indicates the lateral region, which lacks Or22a neurons expressing CYC in *cyc*^{01} flies. The white bars represent EAG responses measured during the light phase at ZT5, and the black bars represent EAG responses taken during the dark phase at ZT17. The cross denotes a significant ($p < 0.00001$) difference in EAG responses between the region in which Or22a neurons are expressing CYC in *cyc*^{01} flies and the region that lacks Or22a neurons expressing CYC in *cyc*^{01} flies at ZT17. The asterisk denotes significant ($p < 0.0001$) differences in EAG responses between the region in which Or22a is expressing CYC in *cyc*^{01} flies at ZT17 and either antennal region at ZT5. Error bars denote SEM. (Reproduced from Tanoue et al., 2004, with permission from Elsevier.)

DISCUSSION AND SUMMARY

Using the utility of CLK and CYC dominant negatives and the versatility of the UAS Gal4 system, we have shown that oscillators within antennal neurons are both necessary and sufficient for olfaction rhythms. This suggests that the hierarchy of organization of oscillators in *Drosophila* is more lateral (peripheral oscillators being equal to the central pacemakers) as opposed to the vertical pattern of organization in the mammalian circadian system. An autonomous oscillator in the olfactory system may best benefit a diurnal organism like *Drosophila,* in which the olfactory system plays a prominent role in courtship, learning and memory (Hall, 1994; Davis, 1996). Also, olfaction rhythms demonstrated by recording EAGs from the antenna, have been described in the cockroach *Leucophaea maderae* also (Page and Koelling, 2003). Interestingly, the rhythm reached a peak in the middle of the subjective night, and unlike what has been described in *Drosophila* here, the rhythm in olfactory responses is dependant on the central pacemakers in the optic lobes, which are entrained by photoreceptors in the compound eye (Page and Koelling, 2003). In mice the olfactory bulb has been shown to possess an independent circadian oscillator that enhances olfactory responsivity each night (Granados-Fuentes et al., 2006). So a pattern emerges here from multiple organisms representing different species, suggesting that the olfaction sensory modality is regulated by the circadian clock to have a heightened response in the subjective night. The ecological consequence of this phenomenon will certainly be a topic of interest to a wide group of biologists. Also, fairly recently, Zhou and co-workers have demonstrated that olfactory behavior in *Drosophila* tested using a T-maze

apparatus in a circadian fashion displays rhythms in behavioral response with maximum score around the middle of the subjective night (Zhou et al., 2005), suggesting the transmission of circadian controlled peripheral processes to higher centers in the brain.

Since oscillators in the antennal sensory neurons are both necessary and sufficient to mediate rhythms in olfaction, it is tempting to speculate that components involved in the olfactory transduction process could be potential targets of oscillator control. These possibilities are the topic of focus in the next chapter.

CHAPTER III

POTENTIAL MOLECULAR TARGETS UNDER CIRCADIAN CONTROL*

INTRODUCTION

The fact that circadian oscillators in antennal sensory neurons are necessary and sufficient for olfaction rhythms implies that components that are part of the olfactory signal transduction cascade could be potential targets for clock regulation. One possibility is that receptor turnover rate could be under clock control with more active signaling units existing at night. Conversely, the clock could suppress olfactory responses during the day. In either case, examining the role of certain molecules that have known functions in regulating receptor function is a logical method of experimental approach. The mechanistic details of olfactory transduction are relatively unclear in *Drosophila*, this fact reduces the number of candidates for experimental testing. Since the *Drosophila* odorant receptors have a unique 'inside-out' topology (Benton et al., 2006), this raises further interesting questions about whether molecules, which have traditional roles in other vertebrate transduction systems, have similar roles in insect olfaction. In this chapter, the focus will be primarily on two functional classes of proteins- arrestin and G-protein coupled Receptor Kinase (GRK or GPRK).

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* Reprinted in part from Ge, H., Krishnan, P., Liu, L., Krishnan, B.,Davis, RL., Hardin, PE and Roman, GW, A *Drosophila* Nonvisual Arrestin Is Required for the Maintenance of Olfactory Sensitivity, Chem. Senses, 2006, 31, 49-62, by permission of Oxford University Press.

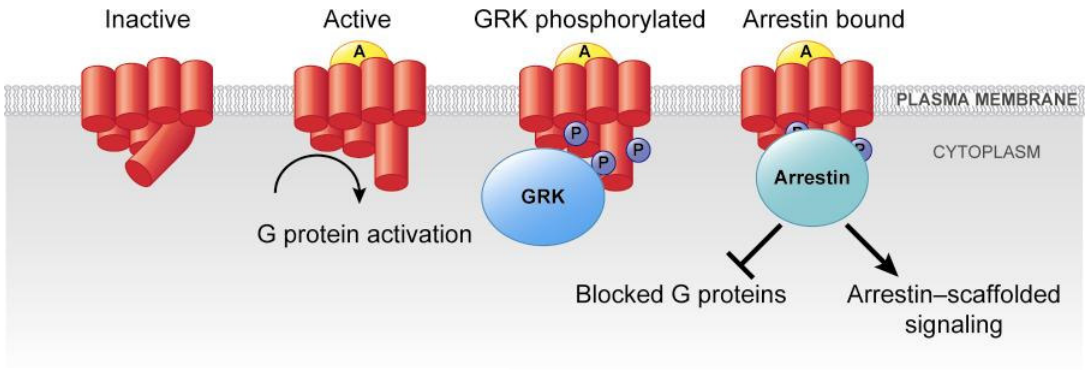

Figure 16: Traditional roles of GRK's and arrestins. Shown is the mechanism of action of these proteins in the desensitization process of G-Protein coupled receptors (GPCRs). See text for details. (Reproduced from Premont and Gainetdinov, 2007.)

These molecules have defined roles in vertebrate transduction systems (Figure 16), the agonist bound receptor triggers a conformational change in the receptor, which facilitates the activation of G proteins by promoting the exchange of GDP for GTP. A G protein coupled receptor kinase (GRK), which also is activated by the change in receptor conformation, binds to the receptor and phosphorylates it. The phosphorylated receptor then binds to an arrestin protein, which couples the receptor to the clathrin coated-pit-internalization pathway. (Premont and Gainetdinov, 2007). This mechanism of desensitization promotes survival of sensory systems by protecting against overstimulation of these cells and making the system sensitive to new stimuli through adaptation (Pitcher et al., 1999).

In *Drosophila*, there are two GPRKs (GPRK1 and GPRK2) (Lee et al., 2004). GPRK-2 protein has a high extent of sequence similarity to members of the mammalian GRK-4 subfamily consisting of GRK-4, GRK-5 and GRK-6, with the exception of a 121 amino acid insertion in the amino terminal region of GPRK-2 (Lannutti and Schneider, 2001). In flies, disruption of the *Gprk-2* gene, results in sterility of mutant females, as a result of reduced levels of egg laying and defective formation of egg structures (Schneider et al., 1997). Also, the *Gprk-2* mutant is defective in cAMP levels and evidence suggests that GPRK-2 functions as part of the cAMP cascade (Lannutti and Schneider, 2001). GPRK1 has been implicated in the modulation of the visual response in *Drosophila* and functions as the rhodopsin kinase (Lee et al., 2004).

There are two well-known visual arrestins and one non-visual arrestin in *Drosophila* viz. *arr1* and *arr2* and *krz* respectively. (Merill et al., 2005; Roman et al.,

2000). Immunolocalization studies show that ARR1 and ARR2 are localized to the ventrolateral surface of maxillary palps, which corresponds to the location of olfactory sensory neurons (Merill et al., 2002). Electropalpogram (EPG) recordings revealed a reduction of about 60% in EPG amplitude to butanol compared to controls in the *arr1* mutant strain (Merill et al., 2002). No defects were found in the *arr2* mutant however. Experiments by Larry Zwiebel and co-workers have revealed decreased peripheral responsiveness (measured by EAG), in flies deficient for *arr1, arr2* or both to a wide panel of chemically distinct odorants (Merill et al., 2005). Behaviorally *arr1* mutants display impaired olfactory avoidance orientation towards attractive odor sources. These deficits vary across intensity and odor concentration. Taken together, these data indicate a clear involvement of arrestins in olfactory function.

The experiments described here will focus on two molecules – a novel non visual arrestin gene , *kurtz (krz)*, which is highly expressed in the antennal sensory neurons as shown in Figure 17 (Ge et al., 2006). Interest in this mutant was also fueled by the observation that *krz* mutants display low olfactory avoidance scores in a behavioral assay (Ge et al., 2006). Hong Ge did the behavioral and immunostaining experiments in Dr. Gregg Roman's lab at Baylor College of Medicine. The *krz* mutant, is a loss of function mutant generated by P-element insertion into the intron of the gene (Roman and Davis, 2000).

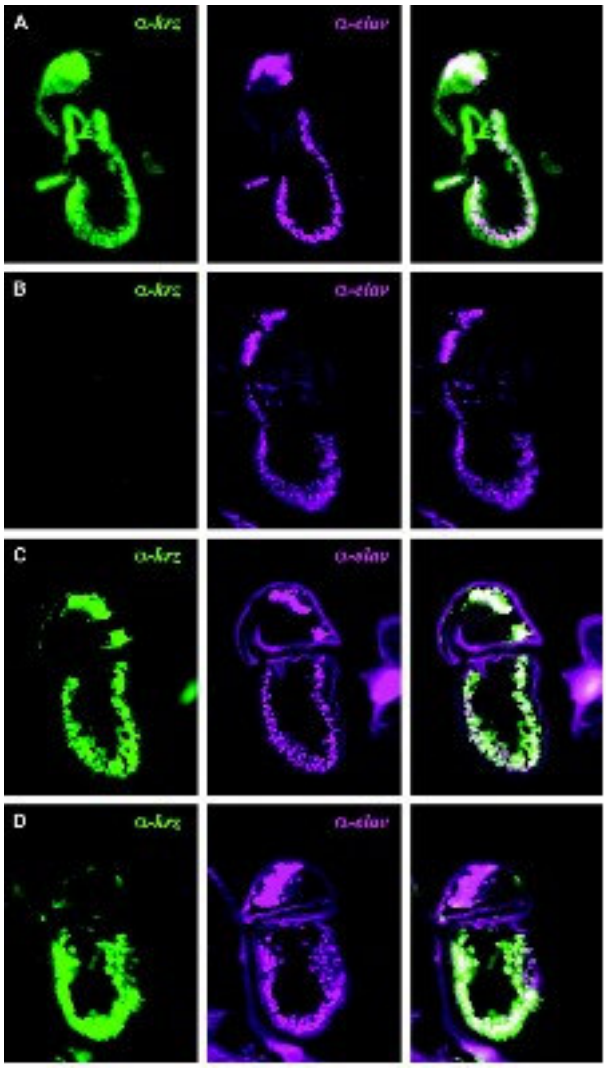

Figure 17: KURTZ is highly expressed in the olfactory system. KURTZ is shown to be expressed in the ORNs in the second and third antennal segments in the wild-type **(A)** (*w1118*), heat shock–rescued **(B)** (*krz1*), pan-neuron–rescued **(C)** (c155; *krz1*), and olfactory receptor–rescued homozygotes **(D)** (*Or83bGal4*, *krz1*). Affinity-purified -KURTZ antibody was detected with goat anti-rabbit–Alexa488 and is shown in green. -elav antibody is a pan-neuron nuclear marker shown in magenta and was detected with goat anti-mouse–Alexa594. Overlapping expression is shown in white. The genotypes of the flies are (A) *w1118* = *w1118*; +; +; (B) *krz1* = P{UAS*krz*}T12/+; *krz1*, P{hspGal4}/*krz1*; (C) c155; *krz1* = c155/+; P{UAS*krz*}T12/+; *krz1*/*krz1*; and (D) Or83bGal4,*krz1* = P{UAS*krz*}T12/+; *krz1*, P{hspGal4}/*krz1*, Or83bGal4 (III). (Reproduced from Ge et al., 2006, with permission of Oxford University Press.)

The *Gprk-2* mutant designated as $Gprk^{6936}$, is a P element insertion located within the 5' untranslated region of the gene (Schneider and Spradling, 1997). This mutant however is not a null mutants as weak mRNA expression can still be detected in the ovaries and CNS tissues (Lannutti and Schneider, 2001).

RESULTS

kurtz Is Required for Normal Odor Induced Receptor Potentials

To address the question of whether *krz* mutants display defective responses in the initial perception and detection of an odorant, EAG responses were measured to quantitate odor induced physiological responses. The krz^{1} homozygotes displayed significantly lower EAG amplitudes to both MCH and OCT than the control hspGal4/+ line. Like the *krz1* behavioral defect, the reduced EAG amplitude was recessive and reversed by the targeted expression of the *krz* cDNA within the ORNs (Figure 18). In summary, the loss-of-*kurtz* function reduces the summed receptor potentials elicited by odors; this defect in the early stages in olfactory transduction can, at least partially, account for the behavioral defects of the *krz1* homozygotes.

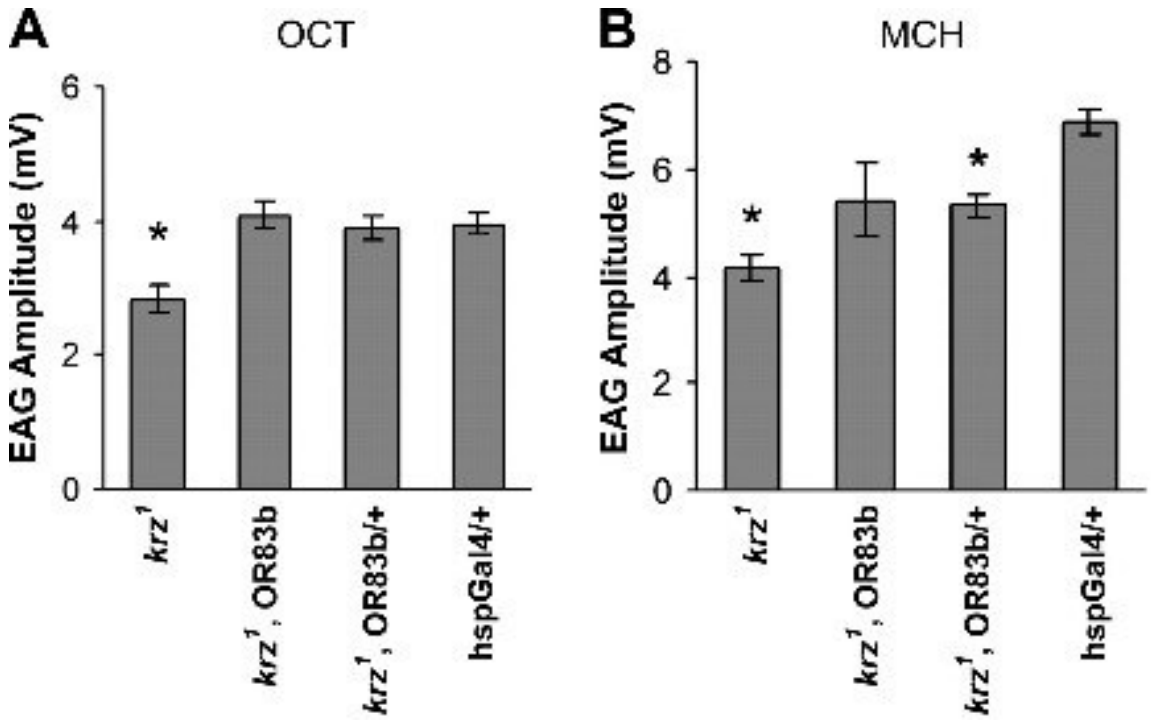

Figure 18: *krz1* homozygotes display reduced receptor potentials. The EAG amplitude of *krz1* homozygotes and control genotype are shown for 0.0001% OCT **(A)** and for 0.01% MCH **(B)**. The genotypes are as follows: *krz1* = (P{UAS*krz*}T12/+; *krz1*, P{hspGal4}/*krz1*); *krz1*, OR83b = (P{UAS*krz*}T12/+; *krz1*, P{hspGal4}/*krz1*, Or83bGal4); *krz1*, OR83b/+ = (P{UAS*krz*}T12/+; P{hspGal4}/*krz1,* P{Or83b-Gal4}); and hspGal4/+ = P{hspGal4}/+. (A) OCT: a significant effect of genotype was found in this experiment ($F = 9.557$, $P < 0.0001$). The OCT-induced EAG amplitude of *krz1* homozygotes was significantly lower than that of the three control genotypes (Bonferroni–Dunn, $P < 0.002$). The three control genotypes were not significantly different from each other. $n = 12$ for each genotype. (B) MCH: a significant effect of genotype was found in this experiment ($F = 28.1$, $P < 0.0001$). The MCH-induced EAG amplitude of *krz1* homozygotes was significantly lower than that of the other three genotypes (Bonferroni–Dunn, $P < 0.0007$). The EAG amplitude of the OR83b, *krz1* heterozygous but not OR83b, and *krz1* homozygous flies was also significantly lower than that of the hspGal4/+ wild-type control flies (Bonferroni–Dunn, $P < 0.0001$). $n = 8$ females for each control genotype and $n = 14$ for the *krz1* homozygotes. (Reproduced from Ge et al., 2006, with permission of Oxford University Press.)

krz Mutants Lack Rhythms in Olfaction and This Deficit Can be Rescued by Cell Specific Expression of _krz_

Next we asked the question whether *krz* has a role in olfaction rhythms. To address this, we measured EAG responses of the *krz* mutant in a circadian fashion. The krz^1 mutants displayed no rhythms in olfaction to ethyl acetate (Figure 19). Interestingly, the amplitude of the response is consistently lower than wild type EAG amplitudes, which are in the order of 8-9 mV. When *krz* levels were increased only in the antennal sensory neurons using Or83b-Gal4 driver and the UAS-*krz* responder, there was a partial rescue of the rhythm in olfaction suggesting that *krz* could have a role in mediating olfaction rhythms (Figure 19).

krz Does Not Have a Major Role in Regulating Olfactory Adaptation Responses

Next, we sought to determine the role of *krz* in regulating olfactory adaptation since *krz* is an arrestin which is known to be involved in bringing about receptor desensitization. In these experiments, a 30-s stimulus duration of odorant was applied to induce adaptation. Following this stimulus, EAGs elicited by a 1-s pulse of the same odorant were recorded at a defined time intervals. Even though the *krz1* homozygotes were less sensitive to odorants in general, they still displayed adaptation of the EAG responses. In these experiments, we found significant differences between the *krz1* homozygotes and control flies with both OCT and MCH. However, the differences were limited and not consistent between odorants. For example, 5 s after the adapting stimulus, the *krz1* homozygotes were significantly less sensitive to OCT, but they were more sensitive to MCH than the control flies. These data suggest that, while *krz* has a

dramatic effect on maintaining olfactory sensitivity, this gene does not have a major role in regulating a decreased sensitivity and recovery rate after strong and persistent exposure to odors. These data are illustrated in Figure 20.

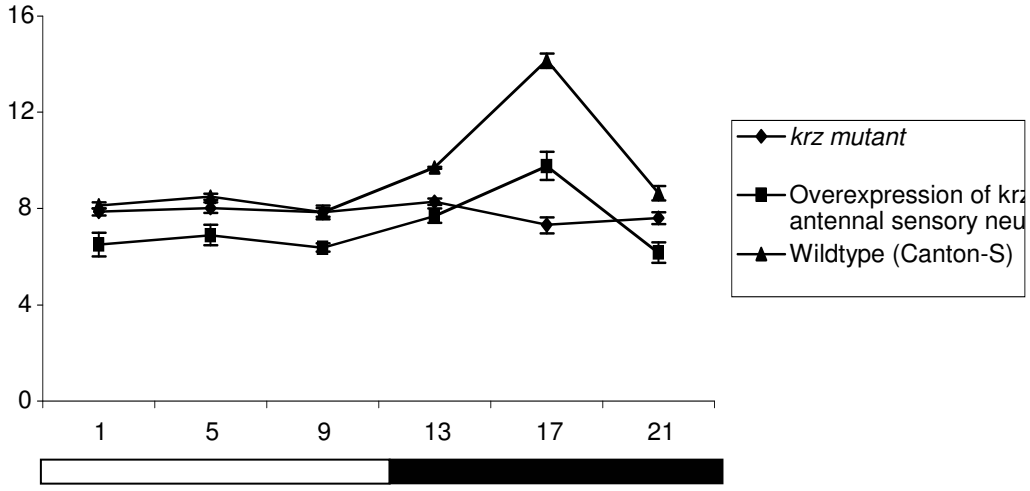

Figure 19: Effect of altering *krz* levels on olfaction rhythms. The white and black bars indicate lights on and off respectively.

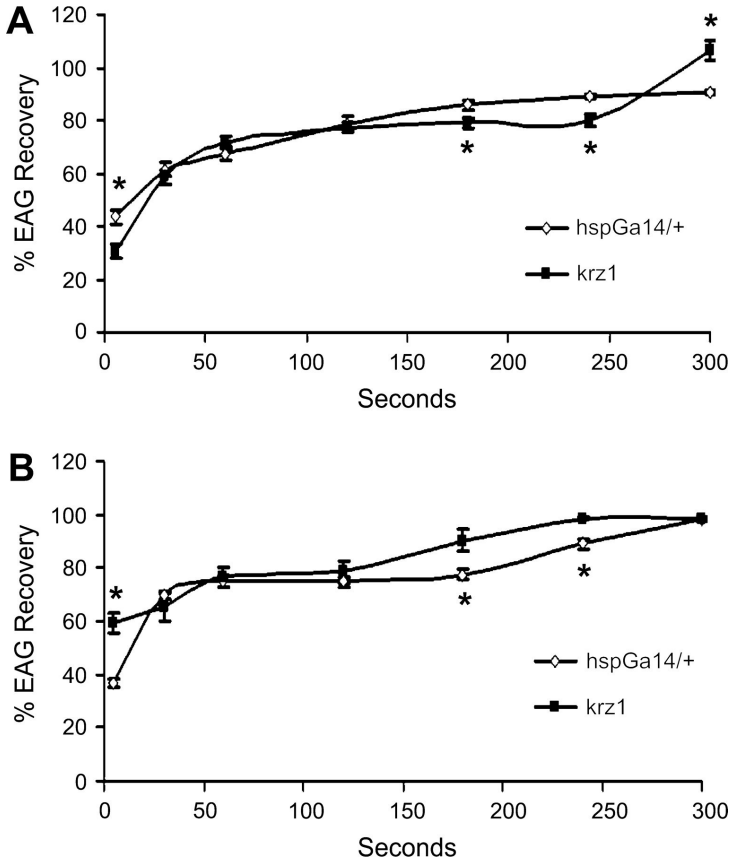

Figure 20: Olfactory adaptation and recovery in *krz*^{1} mutants. The EAGs of *krz1* homozygotes (P{UAS*krz*}T12/+; *krz1*, P{hspGal4}/*krz1*) and hspGal4/+ flies were measured prior to and following a 30-s adapting stimulus of either **(A)** OCT or **(B)** MCH. The percentage of EAG recovery is shown as the amplitude elicited by 1-s pulses of odor at the indicated times following the adapting stimulus. (Reproduced from Ge et al., 2006, with permission of Oxford University Press.)

Altering Levels of GPRK-2 and the Effect on Olfaction Rhythms

To test the influence of GRK-2 levels on olfaction rhythms, EAG responses were measured in $Gprk^{6936}$ flies in a circadian fashion. The responses were similar to the *krz* mutant, with an arrhythmic profile in EAG response to ethyl acetate (Figure 22). When GRK-2 was overexpressed specifically only in the 83b expressing cells, we found loss of rhythms in EAG responses. However, the EAG amplitude of the responses was a constant 13-15 mV in all time points tested characteristic of responses at ZT 17 in the wildtype.

The GPRK-2 Phenotype Persists in a cyc^{01} Background

To determine the effect of the circadian clock on the GPRK-2 phenotype that we obtained in the experiment described in the above section, we overexpresed GPRK-2 in antennal sensory neurons using the Or83b-Gal4 driver in a cyc^{01} mutant background. We found that even in the cyc^{01} mutant background, the EAG amplitudes are constantly high. This suggests that GRK-2 could be involved in the output pathway of the clock mechanism. This result is shown in Figure 23.

Response Kinetics of Wildtype Flies

Since our working hypothesis was concerned with molecules involved in influencing receptor function, we decided to investigate the role of the clock on the recovery kinetics of the EAG response in wildtype flies. The kinetic parameters were calculated using standard procedures described in Alcorta, 1991. Briefly, fall time kinetics was measured as the time taken to decay to 1/3rd of the actual value of the EAG response. This quantitative measure has been widely used as a screen to identify mutants

defective in kinetics of odorant responses. (Merrill et al., 2004; Alcorta, 1991). When we measured fall time kinetics, we did not observe significant differences between ZT 5 and ZT 17 (Figure 21). Also, when a desensitizing odorant stimulus of 30 sec was delivered and recovery monitored over 5, 30, 60 seconds and every minute thereafter, we did not see observable differences in recovery rates at peak and trough time points.

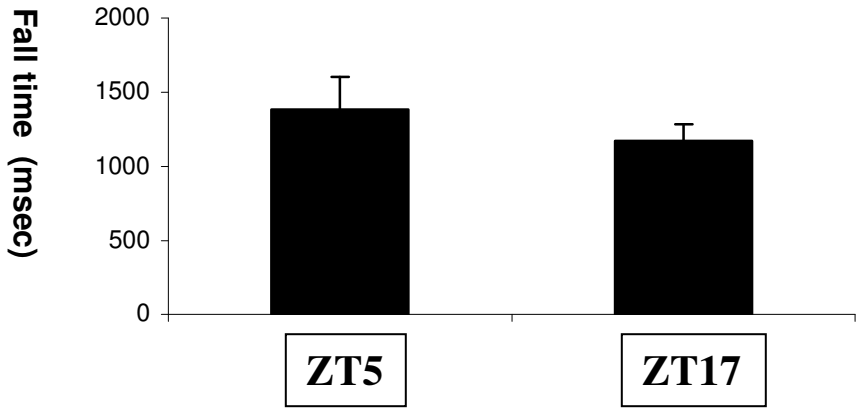

Figure 21: Fall time kinetics of wildtype flies. This is measured as the time to reach 1/3rd of the peak EAG value, is not a parameter influenced by the clock. n = 8 flies per time point.

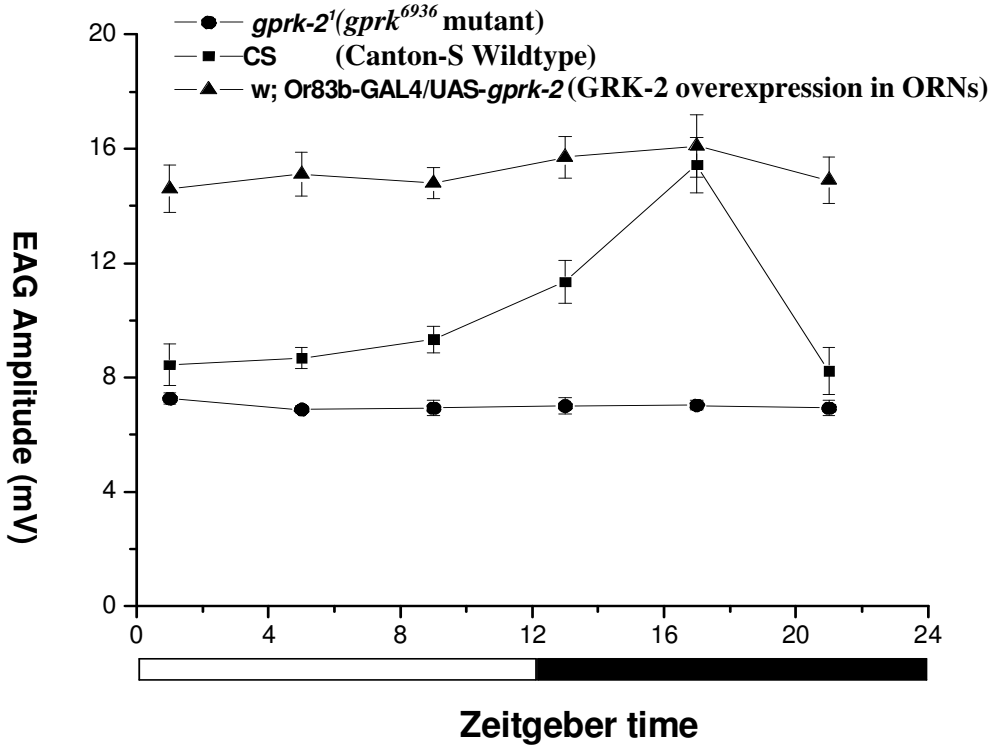

Figure 22: Effect of alteration of GRK-2 levels on olfaction rhythms. n = 12 flies per time point. The overall effects of time of day, genotype and their interaction is statistically significant ($p<0.0001$) by two-way ANOVA for flies overexpressing GPRK-2, Wildtype (CS) and the *Gprk2*^{6936}mutant.

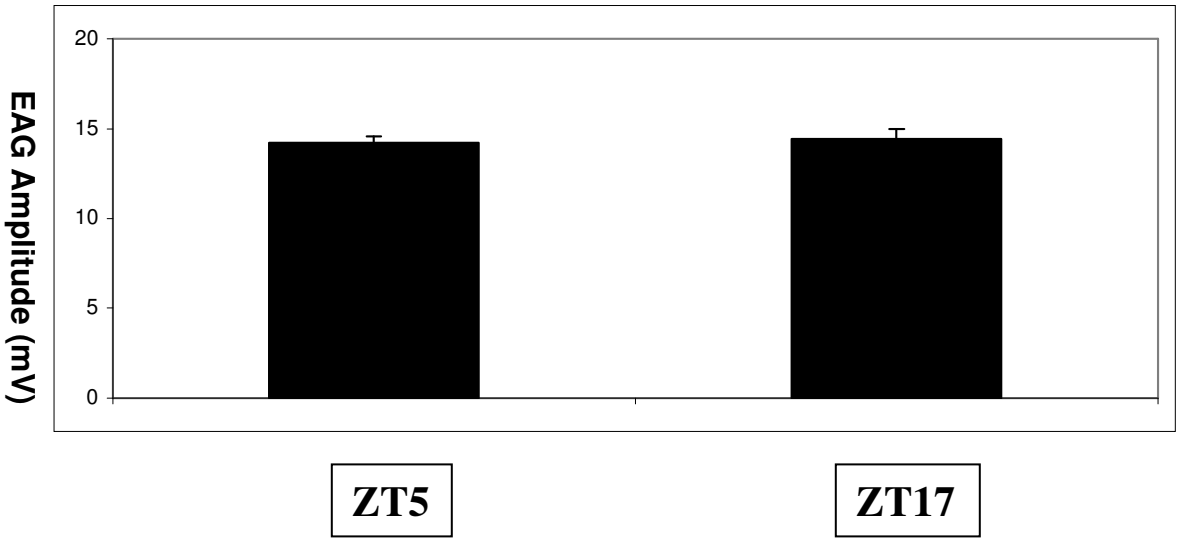

Figure 23: Effect of overexpression of GRK-2 in a *cyc*^{01} mutant background. n = 8 flies per time point

DISCUSSION AND SUMMARY

The results of experiments detailed in this chapter suggest that molecules that have well defined roles in vertebrate transduction cascades are likely to have different functions in modulating olfactory responses in *Drosophila.* If both KURTZ and GPRK-2 were acting in a similar fashion as in vertebrate olfactory systems, then loss of *krz* and *Gprk-2* would lead to increase in olfactory responses. This is not the case in the experiments we have performed in this section. Clearly, both the *krz* and the *Gprk-2* mutant have reduced olfactory responses compared to wildtype.

If *krz* were indeed involved in the desensitization process of odorant receptors, one would expect the *krz* mutants to display a prolonged increase in the activation of downstream effectors, thus resulting in increased olfactory adaptation. However, the *krz* mutants display similar kinetics in desensitization and resensitization of receptor potentials as wildtype controls This suggests that KURTZ is unlikely to act directly on odorant receptors, but could have different role in regulating OR mediated signaling. Also, at the molecular level *krz* mRNA and protein levels are not rhythmic (S. Tanoue, *Personal Communication).* This further suggests that KURTZ acts indirectly on components of the olfactory signal transduction to mediate rhythms in olfaction. Indeed, non-visual arrestins are characterized by the presence of clathrin binding domain and AP2 adaptor binding sites (Ge et al., 2006), suggesting that *krz* could have a rhythmic binding partner. Since *krz* mutant lacks a rhythm in olfactory responses, one other possibility is that KURTZ could be part of the molecular oscillator or in the output pathway.

The data from analyzing the effect of GPRK-2 on olfaction rhythms, reinforces the concept that olfactory transduction is organized differently in flies compared to vertebrate systems. If GPRK-2 were involved in the desensitization process, one would expect the *Gprk-2* mutant to have increased responses to odorants. Conversely, the expectation based on traditional role of GRK-2 would be that overexpression of GRK-2 would lead to decreased odorant signaling. However, both these expectations do not hold true in our experiments (Figure 22). In fact, the observed phenotypes reciprocally contradict the notion that GRKs could be involved in downregulating olfactory signaling.

The recent finding that fly odorant receptors have a unique design (Benton et al., 2006), could be one contributing factor to the phenotypes that has been described above. Also, since the constant high EAG amplitude obtained when GRK is overexpressed in antennal sensory neurons persists in a cyc^{01} mutant, suggests that GRK could be part of the output mechanism of circadian clock function in antennal cells.

In *C.elegans,* animals lacking the *grk-2* gene were found not to be hypersensitive to odorants, but in fact displayed reduced behavioral and physiological responses to odorants (Fukuto et al., 2004). This further strengthens the argument that in invertebrate systems olfactory transduction has a unique design. Also, the loss of the only arrestin in *C.elegans* does not have a consequence on olfactory behavior, suggesting that the reduced olfactory activity seen in *ce-grk-2* mutant could be a result of arrestin independent regulation of odorant receptors.

From experiments described in this section and evidence from other invertebrates, an evolving concept does emerge that in flies odorant transduction is organized in ways that are different from other vertebrate transduction systems.

Interestingly in the visual system of *Drosophila*, members of the arrestin and GRK families are involved in the desensitization of the visual system (Dolph et al., 1993; Lee et al., 2004). In the absence of arrestins, photoreceptors are continuously activated resulting in retinal degeneration (Dolph et al., 1993).

Thus the olfactory system seems to be tuned differently compared to another major sensory system. This further creates an impetus for understanding the unique organization of the olfactory system in flies and to specifically ask the question – What molecular components of the odorant signal transduction pathway are under circadian control and how does the circadian clock interact with the olfaction cascade?

CHAPTER IV

ANALYSIS OF SINGLE UNIT RECORDINGS FROM INDIVIDUAL BASICONIC SENSILLA

INTRODUCTION

Experiments described thus far have shown that antennal sensory neurons possess self-sustainable oscillators for maintenance of olfactory rhythms in the absence of output from central pacemaker cells. Also, flies which have altered levels of an arrestin or GRK-2 show interesting phenotypes which suggests that components of the olfactory signal transduction pathway are potential targets for clock control of olfaction rhythms. To determine cellular processes under circadian control, methods that go beyond the resolution of the EAG are needed. The EAG represents changes in electrical potential between the hemolymph and the antennal surface or in other words, the transepithelial potential. The odor induced EAG response is thought to mainly consist of the summation of receptor potentials of ORNs in close proximity of the recording electrode (Ayer and Carlson, 1992). Also changing the position of the recording electrode results in different EAG responses to the same odorant (Ayer and Carlson, 1992). The EAG method, though useful in many ways, has several pitfalls. The resolution of the response is low and undefined, in other words the number of individual receptor potentials that are being summed is unknown.

These pitfalls can be overcome and, recording single unit responses from an individual sensillum can increase the resolution of the response. In contrast with the

EAG, the parameter being measured in single unit recording is the electrical differences between the sensillar lymph (the recording electrode is placed inside the sensillum- (Figure 24) and the hemolymph. The signal consists of spikes that represent extracellularly recorded action potentials of individual ORNs in the sensillum (Hallem et al., 2004). Spikes can be classified according to their amplitude and in *Drosophila* the larger spike is designated as the A spike and the smaller spikes are classified as B, C and D depending on the number of neurons the sensillum harbors. Single unit measurements will be made from two different basiconic classes – ab1, which harbors four ORNs and the ab3 class which carries 2 different ORNs, which is the case in all other basiconic sensilla except ab1.

The experiments described in this chapter will aim to examine the nature of circadian influence on properties of single unit responses recorded from different large basiconic sensilla. Classification of spikes will be decided based on methods described in de Bryune et al., 2001. Briefly, the size, shape and the frequency of the recorded spikes will be matched to the published data for classification. The parameters that will be studied are 1. Spontaneous firing frequency – which will be also used to classify spike traces to specific sensillar classes. 2. Odor induced frequency in response to a burst of odorant. 3. Spike amplitudes as measured from peak to trough of the spike. Examples of single unit recordings are shown in Figure 25.

MATERIALS AND METHODS

Fly Strains

Entrainment of flies for single unit recording in both LD and DD conditions was done as described (Krishnan et al., 1999). The *Or83b* mutant is a targeted deletion that fails to express Or83b RNA and protein (Larsson et al., 2004). The Δ*halo* mutant is a synthetic deletion that removes both the Or22a and Or22b genes (Dobritsa et al., 2003). The $Gprk^{6936}$ mutant has been described in Chapter III.

Recording of Single Unit Responses

Flies (3-7 days old) were mounted in specially designed apparatus slightly modified from Clyne et al., 1997, in that a fine glass capillary tube was used both to maneuver the antenna on the surface of the cover slip and hold the antenna in place. The antennal surface was observed under 1500X magnification that allowed individual sensilla to be resolved clearly using a BX-51W scope (Olympus). Recordings in the dark was made possible using a filter with a cutoff of <600nm (Leeds). Action potentials were recorded using glass electrodes filled with 0.17M NaCl with tip drawn to <1 μm diameter. The indifferent electrode was inserted into the eye of the fly and the recording electrode was inserted into the base of the sensillum so that the electrode is in contact with the sensillar lymph that bathes the dendrite. These electrodes were positioned using Huxley style micro-manipulators with fine controls (1 μm steps). Signals from the electrodes were fed into a differential amplifier (DP 301, Warner Instruments) and AC signals were recorded (300-10KHz) and amplified 1000X. Recordings were made from at least 3 different ORN's per fly. For all experiments described below a minimum of 4

flies were measured. Single unit recordings were stopped when signs of neuron damage characterized by a high frequency burst of firing was seen. Odorant stimulation was achieved by delivering a quantifiable odor pulse, which interrupts a constant stream of air flowing over the preparation. The number of spikes initiated by the odor pulse was counted manually over 500ms duration. Spike traces were analyzed using Axoscope (Axon) in offline mode and peak to trough amplitudes of individual spikes were computed using software controls. Rate of spike firing was expressed as number of spikes/sec. Examples of single unit spontaneous recordings are shown in Figure 25.

Statistical Analysis

Statistical analysis was done using MS-EXCEL (Microsoft) and Statistica (Statsoft). ANOVA analysis was done using Statistica. Posthoc comparisons were done using Scheffe's test ($\alpha = 0.05$). Student's T-test was used to compare values at peak and trough time points.

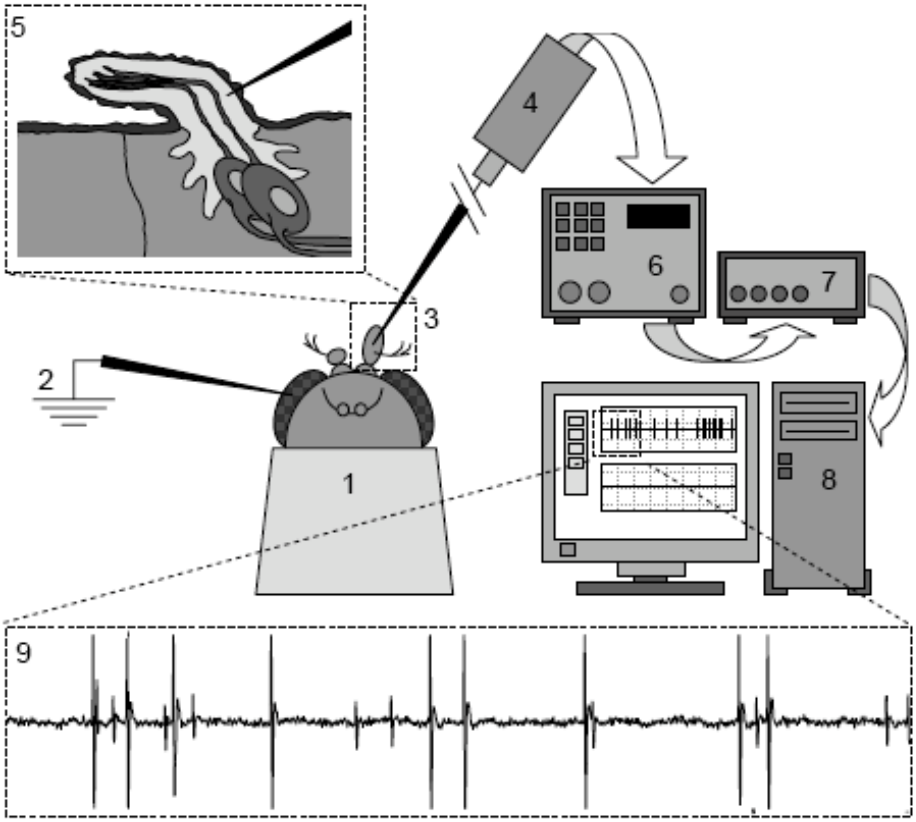

Figure 24: Schematic diagram showing recording of single unit responses. (1) Adult female flies were fixed at the end of a truncated pipette tip so that only the olfactory organs protude out. (2) The ground electrode is inserted into the eye (3). The sensilla on the antennal surface are resolved by the microscope and the (4) electrode is placed in such a way that it pierces the sensillar wall as shown in (5). The signals are amplified (6) and digitized (7) and fed into a computer (8). The traces are analyzed offline by using the Axoscope program (9). (Cartoon adapted from Stensmyr, 2004 with permission.)

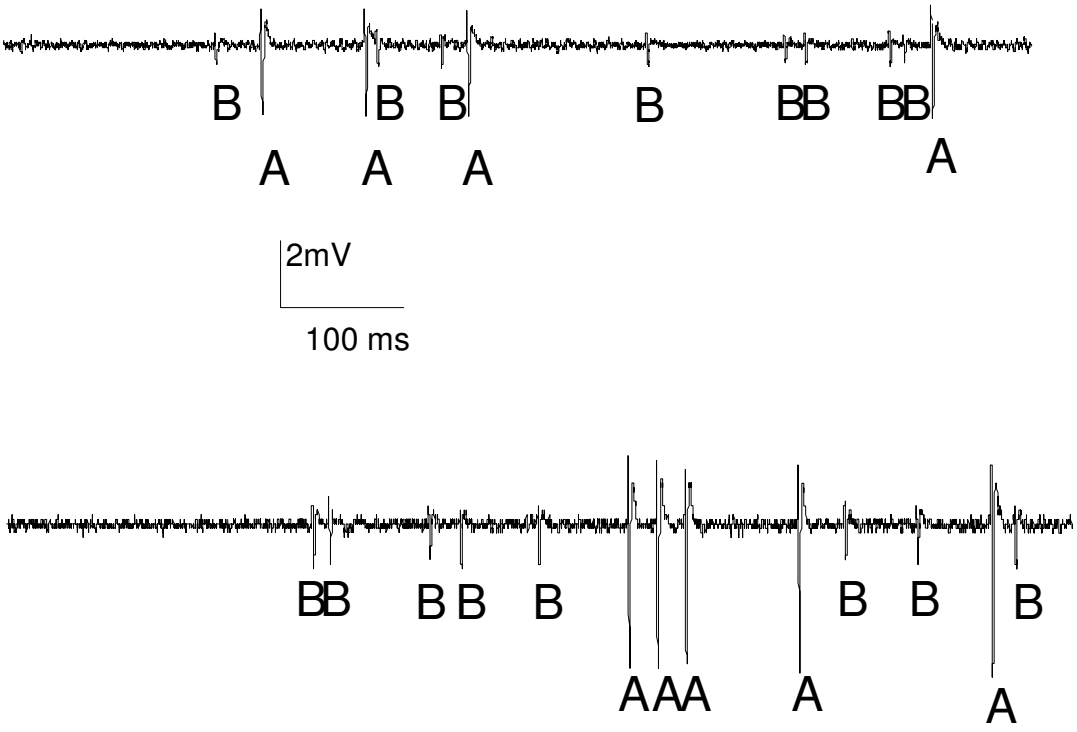

Figure 25: Examples of single unit recordings. Top trace shows spontaneous activity recorded from ab3 sensilla at ZT5. The trace in the bottom shows a recording at ZT17 from ab3 sensilla. A and B denote different classes of neurons.

RESULTS

Spontaneous Spike Amplitude of ab3A and ab3B Neurons Is Under Circadian Clock Control

Circadian oscillators in OSNs of ab3 sensillae are necessary and sufficient to drive EAG rhythms in response to ethyl acetate (Tanoue, 2004). Based on these results, we expected that OSNs in ab3 sensillae would also show rhythms in single unit responses. To determine if this was the case, spontaneous spikes were recorded from ab3 sensillae at times when EAG responses were at their peak (ZT17) or trough (ZT5) values in wild-type flies. At both time points, spontaneous spikes from ab3A and ab3B neurons were seen, but at ZT5 the amplitude of the response from these neurons was lower than at ZT17 (Fig. 26). To determine if this difference represented a diurnal rhythm, spontaneous spike amplitudes from the larger and more easily quantifiable ab3A neurons were measured every four hours during an LD cycle. A significant ($p<0.001$) 2-fold rhythm in ab3A spike amplitude was detected, with a peak at ZT17 and a trough at ZT5 (Fig. 26A). This rhythm in spike amplitude persisted in DD conditions (Figure 26B) and was absent in clock mutants per^{o1} and cyc^{01}, (Figure 26C-D), thus demonstrating that this phenomenon is indeed circadian in nature. When the spike amplitudes of the ab3B neuron were analyzed, a similar rhythm in phase and amplitude to the ab3A neuron was seen (data not shown). These results demonstrate that there is a bonafide circadian rhythm of spontaneous spike amplitude in ab3 sensillae with a phase identical to that seen in EAG responses.

To determine the extent of circadian influence on neuronal firing activity in ab3 sensillae, single unit recordings were made from individual ab3 sensilla and the rate of spontaneous firing and the responses to a quantified pulse (~500ms) of ethyl butyrate was measured in the ab3A neuron. No changes in either spontaneous firing frequency (Figure 26E) or odor induced firing frequency (Figure 26F) were detected at ZT5 or ZT17, indicating that the circadian clock does not influence firing rate in ab3 neurons. We wanted to determine if the clock also controlled odor-induced spike amplitude, but continuous changes in shape of the spike waveform in response to odors precluded quantification of spike amplitude (Clyne et al., 1997; Guillet and Bernard,1972).

Spontaneous Spike Amplitude of ab1A Neuron Is Under Circadian Clock Control

We sought to determine if circadian control of spike amplitude extends to other classes of basiconic sensilla. Single unit recordings from the A neuron of ab1 sensilla were made in wild-type flies collected during 12h light: 12h dark cycles. ab1 sensilla belong to the large basiconic class of sensilla and harbors four ORNs. It has been shown that ab1 sensilla are maximally sensitive to ethyl acetate (de Bruyne et al., 2001), which is the odorant used for most of the EAG experiments described earlier (Krishnan et al., 1999; Tanoue et al., 2004). We found a rhythm in ab1A spike amplitude similar to that seen in ab3 sensillae in that both peak in the middle of the night and have a ~2-fold amplitude (Fig. 27A). This rhythm persists in constant DD conditions and is absent in clock mutants per^{o1} and cyc^{01}, thereby demonstrating circadian clock control (Fig. 27B-D).

When spike amplitudes of the B neuron were analyzed a similar rhythm in phase and amplitude to the A neuron was seen (data not shown), but the responses from C and D neurons were too small to be accurately or reliably measured. These experiments demonstrate that spontaneous spike amplitude is under circadian clock control in two different classes of basiconic sensillae.

We then examined whether the circadian clock influenced spontaneous and odor-induced frequency of spike firing in ab1 sensillae. When spontaneous rate of spike firing of the ab1A neuron was analyzed we found no significant differences across all time points in an LD cycle (Fig. 27E). The same result was seen when ~500 ms pulse of ethyl acetate was applied at different time points throughout the LD cycle and the rate of ab1A spikes were counted during the period of stimulation (Fig. 27F). These experiments demonstrate that spontaneous and odor induced frequency of firing is not influenced by the circadian clock in ab1 and ab3 sensillae, and spike amplitude is the only parameter we measured that was under circadian clock control.

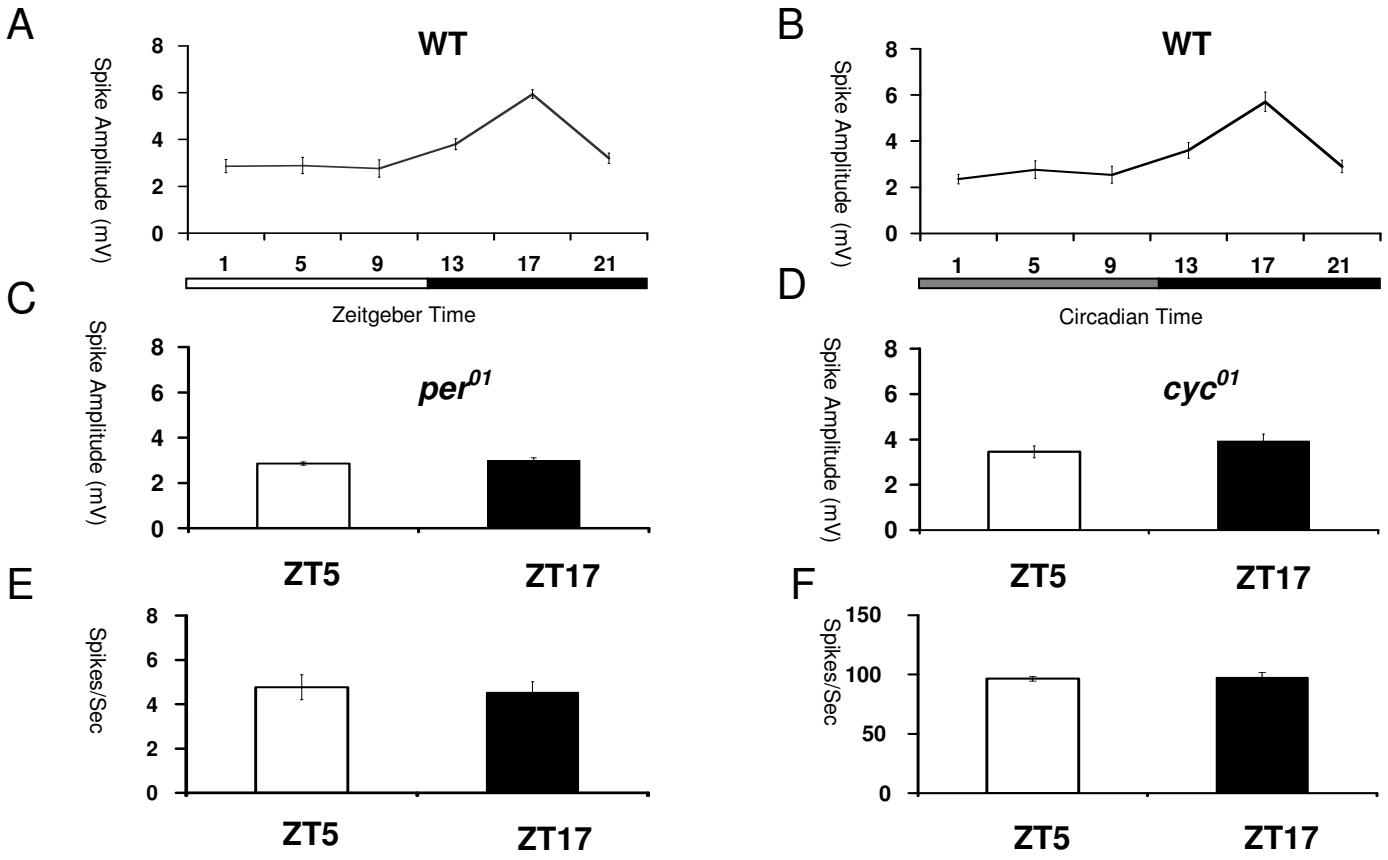

Figure 26: Spontaneous spike amplitudes are under circadian control in the ab3 sensillae. Spontaneous spike amplitudes in wild-type flies during LD cycles (A) or DD (B). The overall effects of time of day is significant by one way ANOVA ($P<0.001$) in panels A and B. (C) Spontaneous spike amplitudes in per^{01} (C) and cyc^{01} (D) flies at ZT 5 and ZT 17. The difference in mean amplitudes of spontaneous spikes at ZT 5 and ZT 17 is not statistically significant ($P>0.4$) in panels C and D. Each time point represents amplitudes calculated from a minimum of 70 individual spikes in panels A-D. (E) Spontaneous firing frequency is not rhythmic in wild-type flies at ZT5 and ZT17 ($P>0.5$). (F) Odor induced firing frequency in response to a 10^{-4} dilution of ethyl butyrate is not rhythmic in wild-type flies at ZT5 and ZT17 ($P>0.4$). Responses from a minimum of 6 OSNs were used to compute firing rate.

Rhythms in Spike Amplitude Are Dependent on Odorant Receptors in Dendrites

An *Or83b* deletion mutant that lacks Or83b mRNA and protein is anosmic because Or83b protein is necessary for localization of ORs to the ORN dendritic membrane in flies (Benton et al., 2006; Larsson et al., 2005). The *Or83b* deletion mutant shows spontaneous activity, but no odor induced responses (Larsson et al., 2005). Since we detect rhythms in spontaneous activity from ab1 and ab3 sensilla, we hypothesized that rhythms in spike amplitude will persist in the *Or83b* mutant. Single unit responses were measured in *Or83b* mutant flies during LD cycles, and ab1A and ab3A spike amplitudes did not show a significant rhythm (Fig. 28A-B). This result argues that certain component(s) of the odorant receptor activated pathway is/are required to sustain the rhythms in spike amplitude. Since *Or83b* mutant flies lack ORs in the dendrites of ORNs, mutants that lack ORs would also be expected to lack rhythms in spontaneous spike amplitude. The Δ *halo* mutant deletes the Or22a and Or22b genes, thereby eliminating odor-induced responses in ab3 sensillae (Dobritsa et al., 2003. When single unit responses of the A neuron in ab3 sensilla were quantified, we found no circadian change in spike amplitude from the Δ *halo* mutant (Fig. 28C).

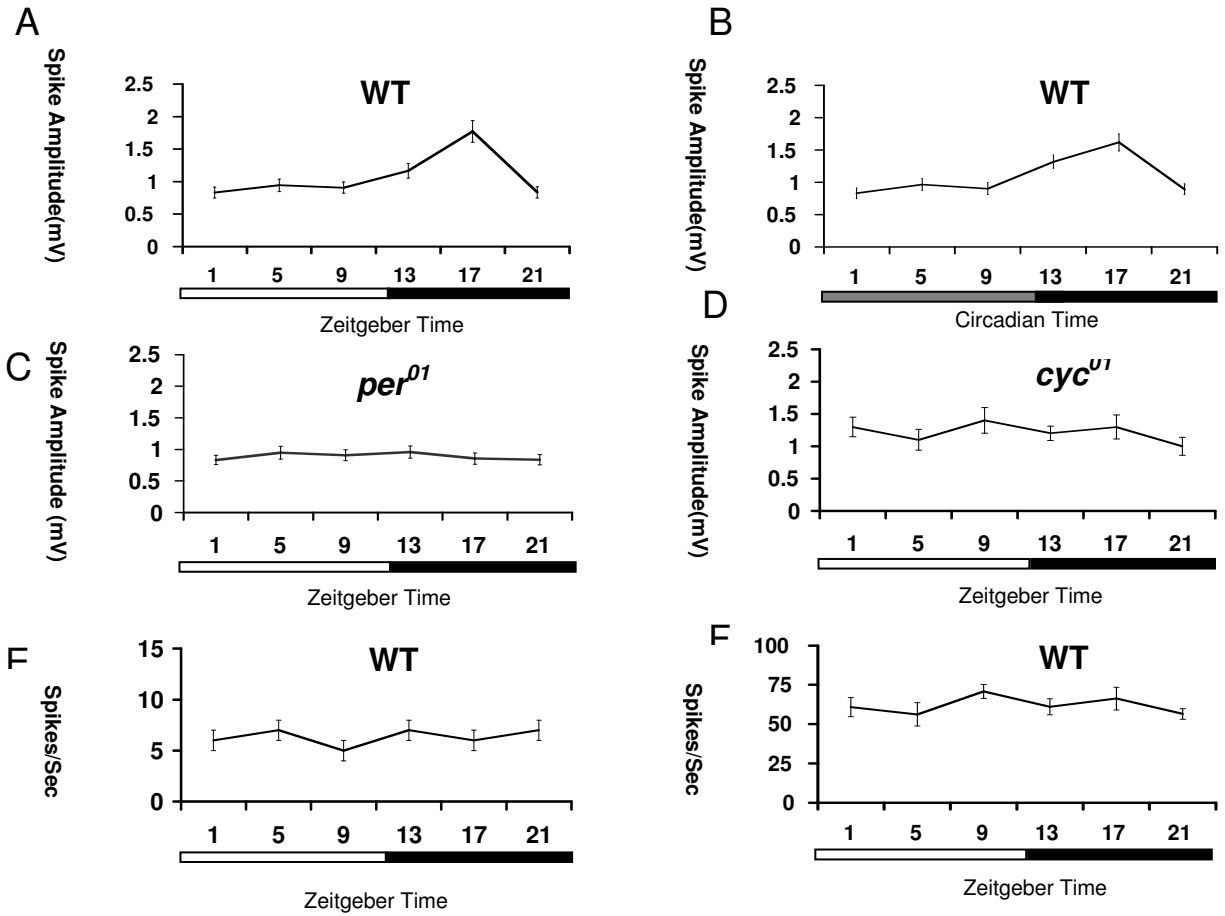

Figure 27: Spontaneous spike amplitudes are under circadian control in the ab1 sensillae. Spontaneous spike amplitudes in wild-type flies during LD cycles (A) or constant darkness (B). The overall effects of time of day is significant by one way ANOVA (P<0.001) in panels A and B. Spontaneous spike amplitudes in per^{01} (C) and cyc^{01} (D) flies during LD cycles. Each time point represents amplitudes calculated from a minimum of 70 individual spikes in panels A-D. (E) Spontaneous firing frequency is not rhythmic in wild-type flies (P>0.4). (F) Odor-induced firing frequency in response to a 10^{-4} dilution of ethyl acetate is not rhythmic in wild-type flies (P>0.4). Responses from a minimum of 6 OSNs were used to compute firing rate.

Effect of Alteration of GPRK-2 Levels on Spike Amplitude Rhythms

When GPRK-2 is overexpressed in antennal sensory neurons using Or83b-Gal4 driver it leads to a constantly high EAG amplitudes at all time points tested, and the GPRK-2 mutant (*Gprk*^{6936}) has trough level responses at all time points tested (Chapter III). Due to the above interesting consequences GPRK-2 levels have on EAG rhythms, we sought to determine the effect of the same on spike amplitude rhythms in different sensillar classes. We observed that the amplitude of the A neuron spike is constantly high at different time points when GPRK-2 is overexpressed in antennal sensory neurons in ab1 and ab3 sensilla. (Figures 29 A-B). When we tested the *Gprk*^{6936} mutant we found lack in spike amplitude rhythms in the A neuron with low values seen in ab1 and ab3 sensilla. (Figures 29A-B). Taken, together, these experiments argue for a common regulation pathway for both EAG rhythms and spike amplitude rhythms in different funicular classes of sensilla.

DISCUSSION AND SUMMARY

Circadian changes in membrane properties have been observed in microbial systems, invertebrates and mammals. Rhythms in spontaneous firing frequency have been described previously in both in isolated *Aplysia* eye (Jacklet, 1969) and in the hamster SCN (Suprachiasmatic nucleus) (Yamazaki et al., 1998). Rhythms in spontaneous activity recorded from the eye of the horseshoe crab, *Limulus,* cycles in opposite phase to the rhythm in activity in response to a stimulus (Barlow et al., 1983). Circadian changes in membrane potential of isolated basal retinal neurons have been documented in the mollusk *Bulla gouldiana* (Michel et al., 1993). In the ciliate protozoan *Paramecium* and in the dinoflagellate, *Gonyaulax* circadian changes in membrane potential have been described (Adamich et al., 1976). Circadian fluctuations in resting membrane potential have also been described in PDF-positive clock neurons in the *Drosophila* brain (Park and Griffith, 2006).

Here we report for the first time circadian changes in spontaneous spike amplitude in single unit responses. We find that spike amplitudes of the spontaneous activity measured from different basiconic sensilla show a circadian rhythm with a peak at ZT 17 for ab1 and ab3 classes sensilla. No circadian changes in either spontaneous or odor induced spike firing frequency were observed in all sensillar classes studied. These rhythms in spike amplitude do not persist in mutants, which lack odorant receptors, suggesting that input from a receptor activated pathway is needed to generate these rhythms.

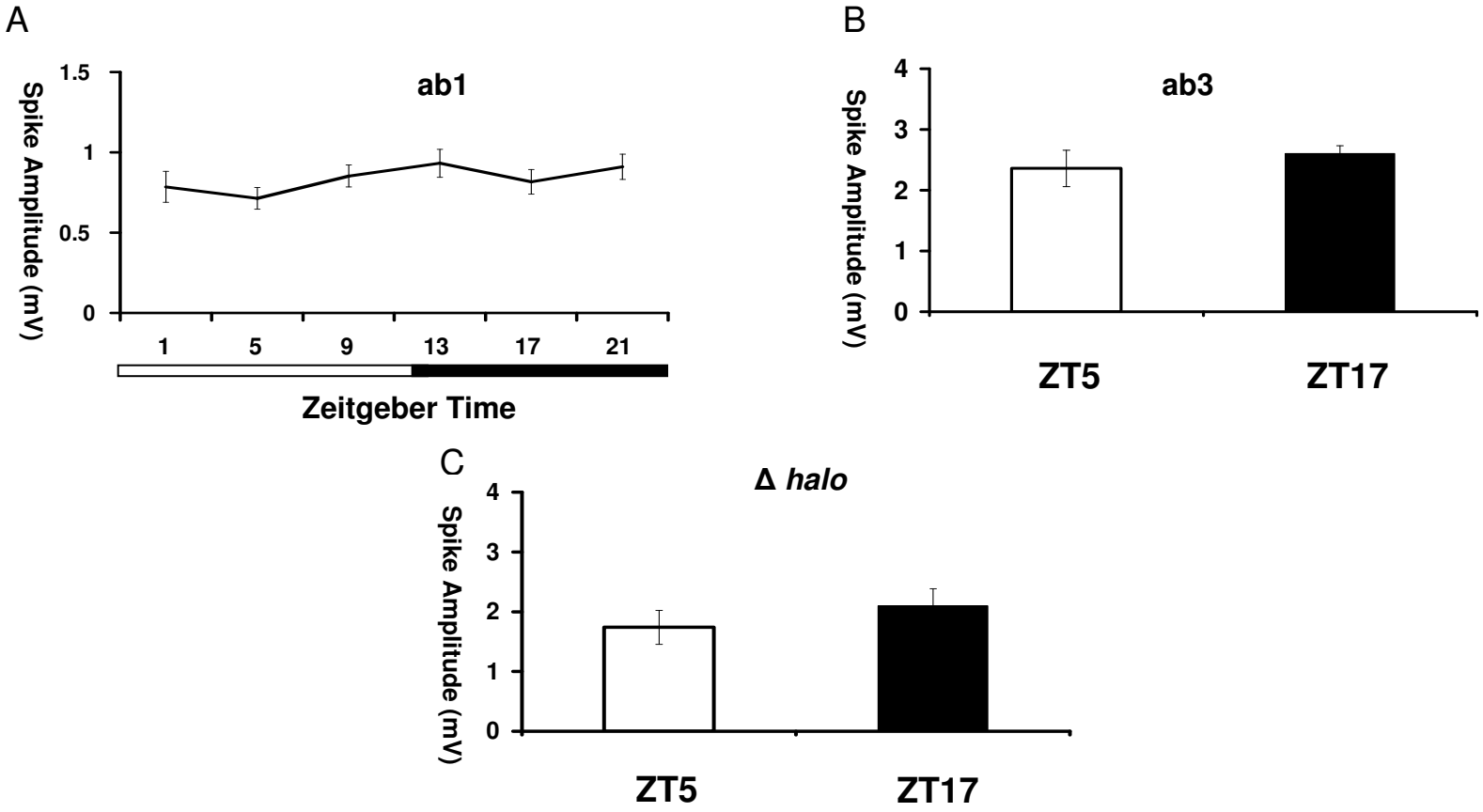

Figure 28: *Or83b* mutant shows no rhythm in spike amplitude of the ab1A neuron and ab3A neuron. Posthoc analysis shows no significant differences in the spike amplitudes of the ab1A or ab3A neuron in *Or83b* mutant flies (A-B). Δ *halo* mutant which lacks odorant receptors 22a/b shows no rhythm in spike amplitude at ZT5 and ZT17 (C). A minimum of 40 spikes was analyzed for each time point in all mutants.

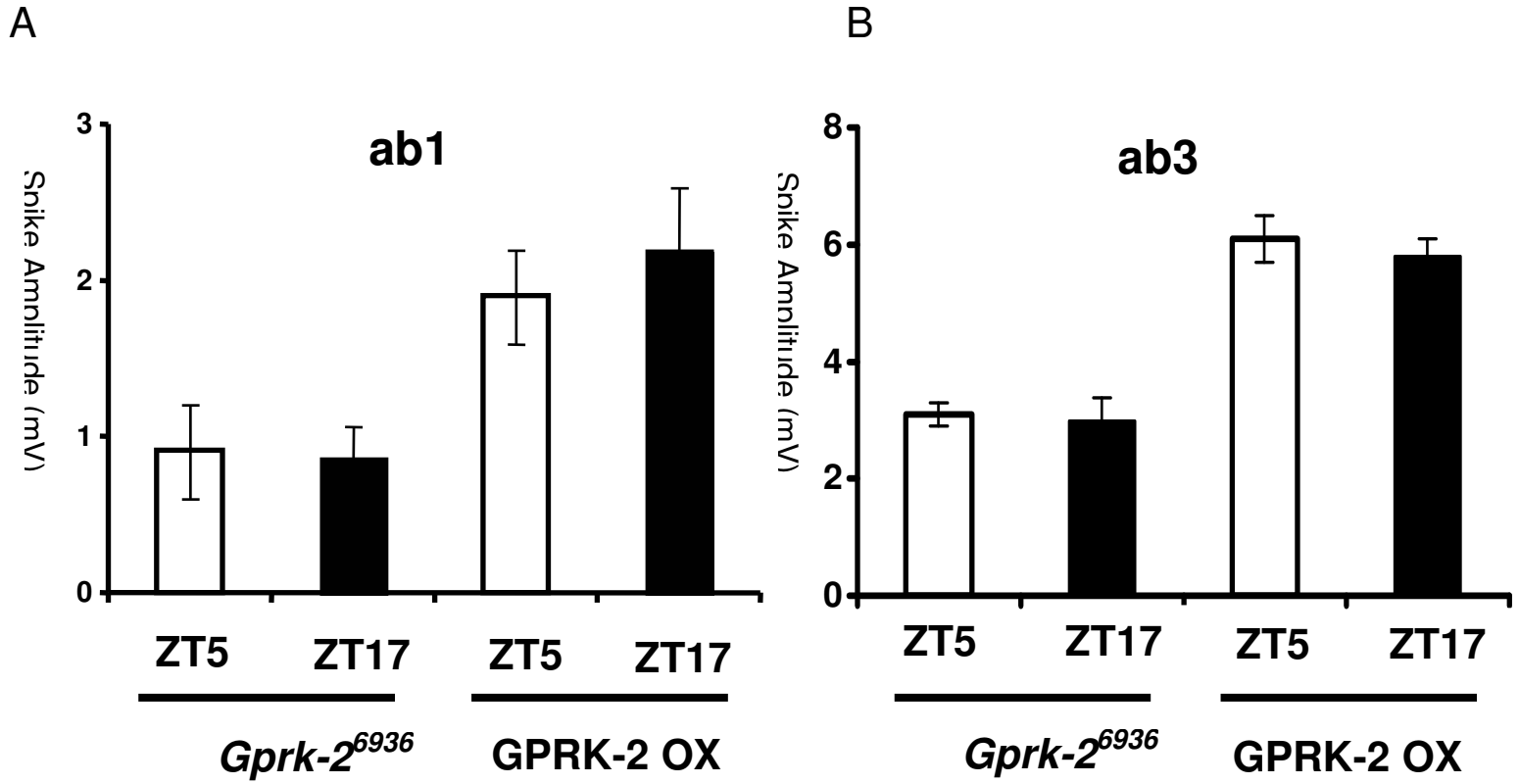

Figure 29: Effect of altering GRK-2 levels on spike amplitude rhythms in ab1 and ab3 sensillae. GPRK-2 OX denotes the overexpression of GRPK-2 in antennal sensory neurons using Or83b-Gal4 driver. A minimum of 40 spikes was analyzed for each time point in all mutants. Mean spike amplitudes at ZT5 and ZT17 for ab1 and ab3 sensillae were not significantly different in Gprk2 mutants and GPRK2 OX flies ($P > 0.1$). Mean spike amplitudes between Gprk2 mutants and GPRK2 OX flies at peak and trough time points was significant in all sensillar classes ($P < 0.0001$).

In insect neurons the spike waveform continuously changes shape during the course of odorant stimulation (Guillet and Bernard,1972). One reason for this phenomenon could be that as the cell approaches its maximum rate of firing, recovery from depolarization is incomplete. Since this odor induced change in waveform drastically alters the spike amplitude of the response, we could not measure with confidence the circadian influence on odor induced spike amplitude. Though a clear explanation of rhythms in spike amplitude are not understood, one could hypothesize that the circadian clock could tune the olfactory system to a higher gain level (higher signal to noise ratio) by modulation of spike amplitude irrespective of the stimulus preferentially in the subjective night.

One hypothesis to explain rhythms in spontaneous spike amplitude is that ion channel activity/composition is under circadian control. Ion channels known to be expressed in the *Drosophila* antenna include DSC1 (*Drosophila* Sodium Channel-1), which shows widespread expression both in the third antennal segment and in the maxillary palps (Kulkarni et al., 2002). PICKPOCKET 25 (PPK25), a member of the DEG/ENaC family of sodium channels, is also expressed in *Drosophila* antenna (Lin et al., 2005). Another possible candidate for clock control is the ether-a-gogo (*eag*) K^+ channel, EAG K^+ channelswhich is thought to be localized to the dendrites of antennal neurons (Dubin et al., 1998). Also, a significantly lower proportion of *eag* neurons responded to ethyl butyrate, a strong activator of the Or22a neuron housed in the ab3 sensillum (Dubin et al., 1998). A regulator of the slowpoke channel, SLOWPOKE BINDING PROTEIN (SLOB), has been shown to cycle in a circadian fashion (Jaramillo

et al., 2004; Sehgal and Levitan,2004). In addition, microarray screens have detected the cycling transcripts of ion channels such as *Shaker*, *trpl* and *slowpoke* in *Drosophila* heads (Ceriani et al., 2002), making these proteins candidate regulators of olfactory rhythms. It is likely that circadian regulation of the same channel(s) and/or regulators give rise to both single unit spike amplitudes and EAG amplitudes, thereby accounting for the similarity in cycling phase in basiconic sensilla. The similar consequences of altering of GPRK-2 levels in antennal sensory neurons on both EAG and spike amplitude rhythms, further fortifies the above mentioned possibility. The fact that GRK-2 directly regulates an epithelial Na^+ channel in isolated salivary duct cells in mice (Dinudom et al., 2004) lends further credence to GPRK-2 being the common mediator of both EAG and spike amplitude rhythms through regulation of an ion channel.

Another potential explanation for rhythms in olfactory electrophysiology are that the composition of the sensillar lymph could be altered in the subjective night in such a way to give larger spike amplitude as well as an elevated EAG value. Precedent for this possibility is seen in moths, where the transepithelial potential is influenced by octopamine and serotonin secretions from accessory cells (Dolzer et al., 2001). Such an explanation would imply that ORNs control accessory cells in the antenna. Single unit recordings from ectopically expressed odorant receptors in the Δ *halo* mutant, which contains an 'empty' Or22a neuron showed that all properties of the ORN response to odorants except for spike amplitude is a property of the receptor, whereas spike amplitude is a property of the individual ORN (Hallem et al., 2004). This also could

mean that certain properties of the cell like membrane dynamics and morphology to name a few could be under circadian influence.

Extending this thought, the size and shape of the cell could be another parameter that is under the circadian clock control. Daily rhythms in cell size have been reported in the fist optic neuropil of *Drosophila*; the axon diameters of L1 and L2 lamina neurons are larger at the beginning of the night and shrink during the daytime (Pyza and Meiertzhagen, 1999. Also, they change shape from an inverted conical form during the day to the cylindrical one at night (Pyza and Meiertzhagen, 1999). Circadian clock-dependent structural changes have also been reported in the *Limulus* eye thereby allowing higher photon capture during the night than day (Barlow et al., 1980). Circadian modulation of spike amplitude contrasts with the situation in the *Limulus* eye, where a higher signal to noise ratio in the night is achieved by suppression of spontaneous activity in the night (Barlow et al., 1984).

Recent evidence demonstrates that locomotor activity of paired male and female *Drosophila* is increased during the subjective night (Fujji et al., 2007). This increased activity has been shown to be dependant on an intact olfactory system. This phenomenon could be one behavioral consequence of an electrophysiological rhythm and could be advantageous to flies in a heterosexual setting, which represents conditions in the wild more accurately than traditional locomotor activity assays. The phase of the peak of basiconics sensilla could translate to heightened behavioral activity throughout the subjective night. Also, Zhou et al., have measured behavioral responses of flies using a T-maze choice assay involving olfactory cues (attractive and repulsive). Using this assay

flies have peak sensitivity to odorants in the middle of the subjective night and low values in the subjective daytime. This rhythm in olfactory responses was shown to be circadian in nature and independent of locomotor activity. (Zhou et al., 2005). This suggests that by a hitherto unknown mechanism information from circadian controlled peripheral processes is transmitted to downstream processing centers. Understanding how rhythms in spike amplitude are ultimately encoded in higher centers of the brain may help us uncover a novel design principle unique to flies.

CHAPTER V

CONCLUSIONS AND GENERAL DISCUSSION

Biological circadian oscillators have evolved in evolutionarily diverse organisms with their internal time keeping mechanisms tightly synchronized with external environmental cues. The circadian system, through control of both period and phase relationship with the entraining zeitgeber, ensures that biological oscillations occurring in diverse tissues are faithfully translated into meaningful physiological and behavioral rhythms. This process aids in the survival of organisms ranging from bacteria to vertebrates. Also the fundamental molecular processes that drive these rhythms remain remarkably conserved across different phylogenetic groups (Yu and Hardin, 2006). Through the work of several eminent scientists in the field of biological timekeeping, especially Colin S. Pittendrigh, several formal properties were established and used in the determination of a bonafide circadian phenomenon as opposed to oscillations which damp and do not self sustain. Formal properties of circadian controlled phenomena are: (1) Entrainment, (2) Temperature compensation, and (3) Ability to sustain a 24hr rhythm in the absence of external input.

The circadian system can be visualized to be composed of three broad functional components: (1) An input pathway, (2) A core molecular oscillator and (3) An output pathway that translates the molecular oscillations into biologically relevant rhythms. In *Drosophila*, the circadian system is composed of interlocked feedback loops in which certain genes are negatively regulated by their own gene products (Hardin, 2005). These

interlocked feedback loops maintain rhythmic cycles in gene expression ultimately driving specific outputs. The mechanistic details of molecular oscillations are fairly well characterized in *Drosophila*. However, little is known about how these molecular cycles translate into rhythmic outputs. The limited number of output pathways known contributes to the lack of understanding of circadian clock function (Hardin, 2005). The most well characterized output rhythm is the locomotor activity of flies, which displays robust rhythms in both LD (Light-Dark) and DD (Dark-Dark) conditions in the subjective dusk. This rhythm requires the rhythmic release of the neuropeptide named PDF (Pigment Dispersing Factor) from sLNvs (small lateral ventral neurons). Another described output rhythm is the rhythm in eclosion of flies. Interestingly, central pacemaker cells are insufficient to sustain this rhythm and that both a peripheral oscillator located in the prothoracic gland and clocks in LNs (Lateral neurons) are required to sustain this rhythm (Myers et al., 2003).

A groundbreaking study from Steve Kay's group in the late nineties (Plautz et al., 1997), caused a paradigm shift in thinking about the organization of circadian clocks in *Drosophila*, and to a large extent this study set the stage for the project described in this dissertation. When GFP (Green Fluorescent Protein) expression was driven by the *period* gene promoter, it revealed the presence of oscillator cells distributed throughout the body of the adult fly. The relationship between these oscillators was studied using the luciferase reporter genes driven by the *per* promoter (*Per-luc)*. Cultured explants from tissues from the antennae, maxillary palp, wings, legs and proboscis rhythmically expressed *Per-luc* under LD and DD conditions in the same phase (Plautz et al., 1997).

This led to the conclusion that light entrainable oscillators are distributed throughout the body of the adult fly and these oscillators are capable of sustaining rhythms in the absence of input from central oscillator cells.

To reiterate the information given in the Introduction section (Chapter I), the adult fly has two primary olfactory organs, the antenna and the maxillary palp. The 3rd antennal segment contains specialized structures called sensilla on its surface, which are the site for primary events in the processing of odor information from the outside environment. Since tissues from these two olfactory organs show autonomous light entrainable oscillators capable of sustaining rhythms in culture, this led to the development of the hypothesis that the olfactory sensory modality in flies is under the influence of circadian controlled processes. One physiological assay to quantify odor evoked responses in the funiculus is an electrophysiological technique called Electroantenogram (EAG). This technique measures the summed receptor potentials of an unknown field in the vicinity of the recording electrode. Indeed, when EAG responses to food odorants like ethyl acetate, were measured in a circadian fashion by Balaji Krishnan, a robust rhythm was revealed which peaks in the middle of the subjective night and has lowest responses during end of the night and during day times (Krishnan et al., 1999). This rhythm persisted in constant DD conditions and was absent in the clock mutants per^{01} and cyc^{01}, proving that this rhythm in EAG responses is a bonafide circadian controlled process. Also, this rhythm persisted in the *per 7.2:2* strain, in which *per* expression is restricted only to certain cells in the central pacemaker neurons and this transgene is not expressed in antennal cells.

This observation suggested that peripheral oscillators are necessary for sustaining this rhythm in olfaction and that central systems sufficient to sustain locomotor rhythms are insufficient to sustain rhythms in olfactory responses. The reason for this heightened sensitivity at night was speculated to facilitate predator detection, opportunistic feeding and/or increase chances of copulation. Moreover, this pattern of elevated responses was observed with regard to two other food odorants iso-amyl acetate and benzaldehyde apart from ethyl acetate. However, a clear explanation was lacking and this rhythm in EAG responses begged the following questions: Which cells are responsible for sustaining this rhythm? Are these rhythms independent of input from central pacemaker cells? If yes, what is the extent of their autonomy?

Although Balaji Krishnan's work showed that central oscillators are dispensable for rhythms in olfaction, they cannot be completely ruled out as insufficient. Just like rhythms in eclosion, the central pacemakers together with the peripheral oscillators in the antenna could mediate rhythms in olfaction, in other words the rhythm could be semi-independent. Other possibilities are these rhythms can be mediated exclusively by peripheral oscillators in antennal olfactory sensory neurons (OSNs). Alternatively, non-neuronal cells like the support cells which surround the OSN's could mediate this rhythm. Among other possibilities, the rhythm in olfaction could be mediated by neurons in the antennal lobe. To tease out these possibilities we have taken advantage of the power and ease of UAS- Gal4 strategies which allows tissue specific expression of a desired gene. The method frequently used to identify cells controlling a rhythmic output is to use cell ablation methods by expression of apoptotic genes. However, in this case

such a method is most likely to undermine the ability of sensory neurons on the antenna to effectively respond to odor cues which can be detected by an electrophysiological assay.

The logical method to address the problem of which cells contribute to maintaining rhythms in olfaction was to use dominant negative forms of CLK and CYC proteins developed by Dr. Shintaro Tanoue. These dominant negative versions lack the DNA binding region and thus are not able to bind to E-box elements and activate transcription of *per* and *tim* genes. These dominant negative constructs were used as UAS responders. Relatively recent strides in uncovering the odorant receptor map of *Drosophila*, largely through efforts of Leslie Vosshall, John Carlson and their respective colleagues, gave us the ability of using odorant receptor coding sequences fused to Gal4 constructs as drivers for cell specific expression in select subsets of olfactory receptor neurons. One such odorant receptor – Or83b is expressed virtually in all sensilla in the antenna. This gave us the ability to drive dominant negative constructs of UAS-CYCΔ and UAS-CLKΔ in a global fashion in antennal sensory cells. Thus the tractability of *Drosophila* as model organism and the availability of tools to manipulate oscillator function in desired cells provided us with an opportunity to address the experimental goals of finding which cells are responsible for rhythms in olfaction and their relative autonomy with respect to central systems. Also, using the then newly developed dominant negative forms of core clock proteins we had a tool to negate oscillator function both in vivo and in vitro systems. We predicted that using this approach would reveal the cells necessary for maintaining rhythms in olfaction and also by silencing the

central pacemaker neurons, we could ascertain whether these peripheral clocks were independent of input from central oscillator cells. Different types of odorant receptors have a distinct pattern of expression on the antenna (Hallem, 2004). This facet gave us the ability to restrict the expression of dominant negative forms to certain groups of cells.

The first experiment to address the above mentioned problem was to drive the expression of both dominant negative forms of CLK and CYC under the influence of the Or83b-Gal4 construct. This experiment revealed that antennal sensory neurons that are positive for the expression of Or83b are necessary for rhythms in olfaction to ethyl acetate (Chapter II), as flies which expressed both the UAS driver and Gal4 driver lacked any rhythms in EAG responses. The EAG measures summed potentials in the vicinity of the recording electrode- so the next question we asked was – would a smaller subset of olfactory neurons expressing the dominant negative forms still lack rhythms in olfaction? We used the Or22a-Gal4 driver to express the mutant clock proteins and found even a small proportion (~30 sensilla) is necessary to sustain the rhythm in olfaction (placing the recording electrode in a different region gave rise to higher EAG amplitudes). These set of experiments showed that oscillators in antennal sensory neurons are necessary to sustain rhythms in olfactory responses.

Antennal oscillators are necessary for rhythms in EAG responses and work by Plautz and co-workers had shown that antennal explants could maintain rhythmicity in culture in the absence of input from central pacemaker systems (Plautz, et al., 1997). Also, previous work suggested that central oscillators are neither necessary nor sufficient

to sustain rhythms in olfactory responses (Krishnan et al., 1999). Taken together this could mean that antennal sensory cells could possess autonomous oscillators which could sustain the EAG rhythms in the absence of input from lateral neurons. We tested this possibility by rescuing wild type CYCLE protein in a cyc^{01} mutant background. In other words, functional oscillator cells were present only in antennal sensory neurons. Flies which had both the UAS-CYC responder and the Or83b-Gal4 driver in a cyc^{01} mutant background showed full rescue of rhythms. When a similar rescue was done only in the Or22a expressing cells alone, rhythms in EAG was restored. These experiments led us to the conclusion that antennal sensory cells possess autonomous oscillator cells fully capable of sustaining the rhythm in olfaction. This is first demonstration of a peripheral oscillator, which is completely independent of input from the lateral neurons in its ability to sustain an output rhythm.

This result shifted the thinking paradigm about there being master oscillators, which sends out input to other peripheral oscillators. This hierarchical model is typical of the mammalian system where the SCN is thought to be the master pacemaker (Yamazaki et al., 2000). Independent clocks in the primary olfactory organ of the fly could best benefit an organism like *Drosophila*, which uses olfactory cues for a host of processes. Perceiving social cues is one example, and since social cues are olfactory in nature and have the power to entrain best illustrates the importance of having independent clocks in the 3rd antennal segment. The olfactory modality is well developed in flies and olfactory cues have shown to be potent stimuli for both associative and non-associate learning tasks (Davis, 1996). Apart from this, normal olfactory behavior is

required for courtship behavior (Hall, 1994). Thus the sense of smell is involved in a host of behaviors that are critical for the survival of the organism. Recent work in Amrein's lab has shown that heterosexual flies display both increased locomotor and courtship activity throughout the subjective night and that this rhythm is dependent on the olfactory system (Fujji et al., 2007). Taken together, an independent clock in the primary olfactory organ of *Drosophila* i.e. the third antennal segment or funiculus serves an important role to increase adaptation to the environment in multiple ways as shown by previous statements. Interestingly, when EAG responses were measured from the antennae of the nocturnal cockroach – *Leucophaea maderae* , the peak in response was during the middle of the subjective day. However, these rhythms in olfaction were dependant on the central pacemaker cells in the optic lobe. This result demonstrates that the organization of the circadian system is different compared to flies and more reminiscent of the hierarchical organization of the mammalian circadian system.

Olfactory systems in other species have also been documented to be under circadian influence. Moths show larger responses to pheromone during the subjective night (Silvegren et al., 2005). Salamanders decrease their foraging responses around early subjective night when exposed to predator extracts (Maerz et al., 2001). In mice olfactory responsivity is highest around the middle of the subjective night (Granados-Fuentes et al., 2006). It is interesting that olfactory sensitivity oscillates in phase with wakefulness in mice and moths, which contrasts with the situation in diurnal flies where peak sensitivity is in the middle of the night. However, in the light of findings from the research of Fujji et al., this notion of considering flies as nocturnal needs to be revisited.

So the grand conclusion from these experiments described so far are that antennal sensory neurons which are the site for early events in olfactory signal transduction cascade, possess autonomous oscillators capable of modulating circadian responses to the food odorant- ethyl acetate. This conclusion opened up many interesting possibilities for further research. The fact that olfactory sensory neurons harbor a host of molecules involved in the initial detection and peripheral processing of odorants makes it attractive to hypothesize that molecules that are involved in mediating the sensitivity of the olfactory system could be potentially under circadian influence. But which molecules expressed in sensory cells in antenna are most likely to be functionally relevant in mediating olfaction rhythms?

We hypothesized that studying the role of molecules involved in the downregualtion or upregulation odorant receptor signaling is the logical way to proceed. Fortunately (or unfortunately) the numbers of experimental candidates to test this hypothesis is few in *Drosophila*, as the odorant transduction pathway in terms of molecular components involved still remains much of mystery. The two top classes of molecules that emerged as potential candidates for experimental study were G-protein coupled receptor kinases (GPRKs) and arrestins, considering their well-known function in modulating vertebrate olfaction transduction systems. GPRKs are known to phosphorylate the agonist bound receptor and this phosphorylated receptor then is a target for arrestin binding and subsequent degradation/recycling of the receptor (Premont and Gainetdinov, 2007). Much of the interest in examining the role of arrestins came from work done in Roman's lab on *kurtz*, a novel non-visual arrestin expressed

ubiquitously in the central nervous system (Roman and Davis, 2000). *krz* mutants display decreased olfactory avoidance responses in a behavioral choice assay and most interestingly *krz* is strongly expressed in the antennal sensory neurons. Also, *krz* mutants display reduced EAG amplitudes to several odorants tested. These evidences together, considering the role of arrestins and the expression profile of *krz*, led us believe that *krz* could have a possible role in olfaction rhythms. Indeed, when EAG measurements were made in circadian fashion, *krz* mutants showed an arrhythmic phenotype with constantly low magnitudes in the range of 6-7 mVs. (Chapter III). Since the behavioral and electrophysiological deficit of the *krz* mutant could be rescued by targeted expression of *krz* in post developmental ORNs, we asked the question whether expression of *krz* in the antennal sensory neurons is sufficient to restore olfaction rhythms. When Or83b-Gal4 was used to express UAS-*krz*, in the antennal sensory neurons a partial rescue of the rhythm in olfaction was observed. However, unpublished work done by Dr. Shintaro Tanoue showed that neither *krz* protein levels nor its mRNA has any circadian pattern of expression. This suggests that *krz* is not the rhythmic molecule which interacts with components of the olfactory signal transduction cascade, but could have an indirect role by binding to other rhythmic molecule(s). Another possibility is that *krz* could be part of the circadian clock interacting with specific component(s) involved in sustaining molecular oscillations of clock proteins. Also, *krz* has no major role in recovery after desensitization cased by a persistent exposure odorant stimulus. Taken together, this argues for a role for *krz,* which belongs to the arrestin family, to most likely have different role in olfactory transduction as

opposed to vertebrate olfactory transduction systems. Another line of evidence to support this line of thinking is the fact that olfactory sensilla are elongated structures with internal diameters in the range of 50nm in case of large basiconic sensilla (Ge et al., 2006). This size range precludes the formation of the molecular machinery needed for the internalization of odorant receptors (Ge et al., 2006).

Another class of molecules, which are involved in the regulation of receptor levels, are the GPRKs regulate receptor levels by making them targets for arrestin mediated degradation. Given the role of GRK in its traditional sense and the results we obtained with the *krz* mutant, it was convenient for us to hypothesize that GRKs also have a role in mediating olfaction rhythms. When we tested the GRK mutant designated as designated as $Gprk^{6936}$, we observed lack in olfaction rhythms in EAG amplitude. The next question was- can this deficit in rhythm be restored by targeted expression of GPRK only in Or83b expressing cells? This experiment yielded a very surprising result and has fueled much of our interest in GRK as a potential mediator of olfaction rhythms. Targeted expression of GRK in antennal OSNs yielded no rhythms in olfaction. However, the EAG amplitudes were in the range of 14-15 mVs at all times points tested throughout the zeitgeber cycle. One shall recall at this point that these amplitudes are characteristic at ZT 17 or in the middle of the subjective night in the wild type fly. This surprising result led us to believe that altering GRK levels could have profound effects on EAG amplitude and thus gave us the impetus for focusing on GRK as a potential target. What made the results most interesting was that it completely reversed the expected role of GRK (from its function in vertebrate systems). This, together with the

arrestin (kurtz) data, gave us a new way about thinking about the roles of certain molecules which have well defined roles in vertebrate olfaction but are likely to have different roles in *Drosophila* olfactory transduction. Our views were fortified by the work of Fukuto and colleagues, who showed that *C.elegans* lacking the grk-2 gene (*ce-grk-2*), showed reduced behavioral responses and did not display hypersensitivity (the expected result if GRK were indeed acting the same way as it did in vertebrate olfaction). Also, loss of the only arrestin found in worms (*arrestin-1*) has no profound effect on olfactory responses suggesting that odorant receptors could be down regulated in an arrestin independent manner (Fukuto et al., 2004). Recent evidence from Leslie Vosshall and colleagues indicates that *Drosophila* odorant receptors have a unique cellular topology, with their N termini inside the cell (Benton et al., 2006). Could this be the cause of molecules like arrestins and GRKs having altered function in fly olfactory system? The consequences of this altered topology on downstream transduction events is a topic that needs much experimental attention to ultimately interpret phenotypes observed with alteration of select molecules of the transduction cascade.

All the experiments described above have been done using the electroantennogram (EAG) technique. Though EAG is a convenient assay for studying olfactory phenotypes, it has one serious drawback. The EAG represents changes in electrical potential between the hemolymph and the antennal surface or in other words, the transepithelial potential. The odor induced EAG response is thought to mainly consist of the summation of receptor potentials of ORNs in close proximity of the recording electrode (Ayer and Carlson, 1992). The number of individual receptor

potentials that are being summated however is unknown. Thus the EAG lacks resolution and this fact limits the extent of interpretation of data obtained by testing mutant strains deficient in some aspect of olfactory signaling. To remedy this drawback, we decided to measure single unit responses from individual sensillae. Singe units are an extracellular measure of action potentials arising from individual ORNs (de Bruyne et al., 2001). The number of spikes classes defined by its amplitude can discern the number of neurons housed inside a sensillum.

Using glass microelectrodes drawn to >1µm, we measured single unit responses from individual basiconic sensillae viz. ab1 and ab3, both belonging to the large basiconic class of sensillae (de Bryune et al., 2001) (Chapter IV). We decided to measure both amplitude of the spike and frequency, as these are two most discernible parameters seen under visual eye while making single unit recordings. When these recordings were made in a circadian fashion, we found that the only parameter that was under the influence of the circadian clock was the spike amplitude of the spontaneous activity. This change in spike amplitude persisted under constant DD conditions and no rhythms were observed in clock mutants per^{01} and cyc^{01}. All analysis was done on the A spike as it is the largest spike and hence the most reliable to quantify. However, we did observe a rhythm in the amplitude of the B spike as well (data not shown). The other classes of spikes namely C and D from the ab1 neuron were too small to quantify accurately. Interestingly, the phase of the peak in response was similar to that of the EAG rhythm in that the peak was in the middle of the subjective night and the difference in amplitude (peak to trough) was ~2 fold in magnitude just like the rhythm in EAG

responses. Apart from spike amplitude, we also assayed spontaneous frequency and odor induced frequency. We found no change in these two parameters with constant frequency of firing at all time points tested. In insect neurons the spike waveform continuously changes shape during the course of odorant stimulation (Guillet and Bernard, 1972). One reason for this phenomenon could be that as the cell approaches its maximum rate of firing, recovery from depolarization is incomplete. Since this odor-induced change in waveform drastically alters the spike amplitude of the response, we could not measure with confidence the circadian influence on odor induced spike amplitude. The same pattern of circadian influence on spike amplitude was seen in the ab3 neurons with no change in frequency of both spontaneous or odor evoked responses.

It has been reported by both Leslie Vosshall and John Carlson and their respective colleagues, that mutants which fail to localize receptors to the dendrite still have spontaneous activity (Larsson et al., 2005; Dobritsa et al., 2003). We mused that since these rhythms were seen in spontaneous spike amplitude, maybe these rhythms are independent of input from the receptor. When we tested the *Or83b* mutant, in which odorant receptors fail to localize to the dendrites as *Or83b* acts a chaperone to ensure proper OR function, we found no rhythms in spike amplitude in this mutant. We then asked the question if deleting only the bonafide odorant receptors leads to loss in rhythms of spike amplitude. Indeed, loss of Or22a/b in the Δ*halo* mutant had no rhythms in spike amplitude. This led us to believe that some component(s) of the OR activated signaling pathway is needed to sustain this rhythm in spike amplitude.

We also tested the effect of alteration of GRK levels on spike amplitude rhythms. We obtained constantly high spike amplitudes (ZT 5 and ZT 17) when GRK was over expressed and in the GRK mutant there was no rhythm and the amplitudes were near the trough values seen in wildtype. Another possible explanation for this result is that the composition of the sensillar lymph could be altered at night in such a way to give larger spike amplitude as well as an elevated EAG value. There is precedence for this school of thinking- in moths the transepithelial potential is influenced by octopamine and serotonin secretions from accessory cells (Dolzer et al., 2001). Research from John Carlson's lab has shown that spike amplitude is a property of the cell and not the receptor – ectopically expressed receptors have properties of the individual ORN rather than the specific receptor (Hallem et al., 2004). This means that properties of the cell could be under circadian influence. Extending this thought, the size and shape of the cell could be one parameter that could be under the control of a pacemaker. This concept has precedence as well. Daily rhythms have been reported in the fist optic neuropil of *Drosophila*. Two cells L1 and L2's axons have larger sizes at the beginning of the night and shrink during the daytime. Also, they change shape from an inverted conical form during the day to the cylindrical one at night (Pyza and Meiertzhagen, 1999). Structural changes in a circadian fashion have also been reported in the *Limulus* eye which allows higher photon capture during the night than day. During subjective day, these structural changes reverse: elongation of both aperture and rhabdome, reducing the number of photons captured (Barlow et al., 1980).

In summary, in this dissertation work we have shown that, antennal sensory neurons possess independent oscillator cells that are both necessary and sufficient for rhythms in olfactory rhythms to ethyl acetate. We have shown this by exploiting the UAS-Gal4 approach by expressing dominant negative forms of CLK and CYC in antennal sensory neurons using odorant receptor-Gal4 drivers. This implies that molecules of the odorant signal transduction pathway could be potential targets for clock control. We did find very interesting phenotypes with *krz*, a non-visual arrestin and GRK-2. These phenotypes argue for roles of these molecules that are very different from their well-defined roles in vertebrate olfaction. This gave us a new insight into looking at new roles for 'old' molecules. Finally, we have shown that single unit responses recorded from individual basiconic sensilla show a rhythm in spontaneous spike amplitude in both ab1 and ab3 classes of sensilla. This rhythm was found not to be independent of input from the odorant receptor activated pathway. However, no rhythms were found in either spontaneous or odor evoked frequency. We still do not have a clear explanation to whether the oscillations in spike amplitude are the basis for EAG rhythms.

FUTURE DIRECTIONS

Understanding how rhythms in olfaction translate into meaningful behavioral patterns is a key question, which will of much interest to a wide range of biologists ranging from ecologists to neurophysiologists. In the light of work from Amrein's lab, one behavioral consequence of this rhythm in olfaction could be heightened locomotor and courtship activity in the subjective night (Fujji et al., 2007). How is this heightened

sensitivity brought about? What are the contributions individual sensillar classes to this behavioral pattern? These are some interesting questions that will shed light on olfaction rhythms at a more holistic level. Chalking out the nuts and bolts of the olfactory signal transduction cascade in flies will be a major thrust for research. This exploration is crucial in understanding and interpreting data obtained with several mutant strains. What are the consequences of altered 'inside-out' membrane topology? What are the roles of KRZ and GRK specifically in terms of their mechanisms of action? Since spike amplitudes are elevated during subjective night, what molecules could be influencing this phenomenon? A microarray analysis is called for to understand cycling transcripts in the 3rd antennal segment. Behaviorally, flies show a robust rhythm in responses to odorants in a T- maze apparatus, which peaks in the subjective night (Zhou et al., 2005). This rhythm is dependant on clock genes and is independent of locomotor activity. As earlier mentioned locomotor activity of heterosexual paired flies displays a heightened activity throughout the subjective night (Fujji et al., 2007). This increase in activity has been shown to depend on an intact olfactory system. This suggests that by hitherto unknown mechanism peripheral information of circadian controlled olfactory responses are transmitted to higher brain centers. How are rhythms in spike amplitude encoded and by what pathway are major questions that beg an answer. Thus new insights into mechanistic details these phenomena might emerge with more directed research in the above mentioned fronts.

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